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Isolation and Characterization of Microorganisms with Probiotic Potential

Mestrado em Microbiologia Aplicada

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Abstract

Probiotics are live microorganisms which, when administered in adequate amounts, confer health benefits to the host. Various benefits have been described, particularly those pertaining to improvements in gastrointestinal diseases.

Probiotics may be isolated from several sources, one of these being vegetable products. Sauerkraut is a fermented cabbage product, resulting from fermentation by a lactic acid bacteria microbial succession, and therefore it can be a potential source of probiotic candidates. Nonetheless, several characteristics should be addressed in order to consider the use of a microorganism as a probiotic, including its safety, resistance to gastrointestinal tract conditions and ability to produce a health effect on the host.

The objective of the present work was the isolation and characterization of probiotic microorganisms from sauerkraut fermentations. Isolated microorganisms were characterized phenotypically and genotypically, identified to the genus level and their safety and probiotic potential was evaluated. Furthermore, *Caenorhabditis elegans* was used as an animal model to analyze the probiotic potential of selected bacteria.

Sauerkraut fermentations developed successfully and 114 isolates, originating from four different fermentation recipes, were further characterized. After phenotypic and genotypic analysis, representative isolates were chosen, 52% of these being *Lactobacillus* spp. and 33% *Leuconostoc* spp. LAB diversity and composition throughout fermentation differed depending on the type of cabbage used as substrate for sauerkraut production. Only one isolate presented β-hemolysis, and 42% were resistant to at least one antimicrobial compound associated with transmissibility to other microorganisms. Regarding resistance to gastrointestinal conditions, 88% of isolates were resistant to bile and 20% to low pH. Six probiotic candidates were further studied, all of them possessing antimicrobial activity against *Listeria monocytogenes* and three being resistant to lower pH values than previously tested. Two of the best candidate isolates were tested using the *C. elegans* model, but did not present a protective effect against *Serratia* sp. Instead, a synergistic effect was observed, increasing its pathogenicity.

Our results confirmed the applicability of sauerkraut fermentations as a source for probiotic isolation. Three candidates were isolated from these fermentations, and although two of them did not show probiotic characteristics in the *C. elegans* model tests, further work must be done in order to fully characterize these isolates and clarify their probiotic potential.

Keywords: probiotics; lactic acid bacteria; sauerkraut; *Caenorhabditis elegans*

Resumo

A microbiota intestinal define-se como a totalidade de microrganismos que se encontram no trato gastrointestinal, sendo a sua composição variável de indivíduo para indivíduo. Esta comunidade microbiana assume um papel preponderante na saúde humana, pois influencia o hospedeiro ao nível metabólico, nutricional, fisiológico e imunológico. A perturbação da microbiota normal, denominada disbiose, está associada a várias doenças. Desta forma, torna-se importante modular esta microbiota, de forma a prevenir ou tratar eventuais episódios de doença. Uma possível abordagem é através da ingestão de produtos probióticos.

Probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro. A maioria destes microrganismos pertencem ao grupo das bactérias lácticas, principalmente aos géneros *Lactobacillus* e *Bifidobacterium*, e podem ser incluídos em vários tipos de produtos alimentares ou suplementos, nomeadamente em produtos lácteos fermentados como os iogurtes, ou alternativamente em vegetais, produtos baseados em cereais, entre outros.

Os probióticos podem ser isolados de várias fontes, sendo as principais os produtos lácteos e o trato intestinal humano. No entanto, outros produtos fermentados, como por exemplo os baseados em vegetais, também podem ser fontes importantes para o seu isolamento. O chucrute é um destes produtos, resultando da fermentação espontânea de couve após a adição de sal. Esta fermentação é caracterizada por uma sucessão microbiana que pode ser dividida em duas etapas. A primeira apresenta uma maior quantidade de microrganismos heterofermentativos, enquanto a segunda apresenta um maior número de organismos homofermentativos. As espécies *Leuconostoc mesenteroides* e *Lactobacillus plantarum* são as mais comuns durante a fermentação do chucrute, apesar de já terem sido observadas outras espécies bacterianas, em quantidades mais reduzidas.

Várias características devem ser consideradas na seleção de microrganismos probióticos, sendo que estas são normalmente específicas de cada estirpe. Primeiro, é necessário avaliar o perfil de segurança dos candidatos a probióticos em relação ao organismo humano, existindo poucos casos de probióticos prejudiciais para a saúde humana. Outra questão importante é a presença de genes de resistência a compostos antimicrobianos que possam ser transmissíveis a outros microrganismos. Para além disto, os candidatos devem conseguir sobreviver às condições rigorosas presentes no trato gastrointestinal, tais como o baixo pH e a presença de bílis e enzimas digestivas, e possuir capacidade de adesão a este órgão, de forma a que o possam colonizar eficazmente. Por fim, estes microrganismos devem produzir um efeito benéfico para o organismo humano.

Vários benefícios decorrentes da utilização de probióticos já foram descritos para o tratamento e prevenção de doenças gastrointestinais, como é o caso da diarreia associada a antibióticos, a gastroenterite infeciosa, algumas doenças inflamatórias intestinais, entre outros. Além disso, também já foi observado um efeito benéfico no tratamento de infeções do trato respiratório. Outros benefícios para a saúde humana necessitam de mais provas para que sejam

comprovados, como por exemplo o tratamento de infeções causadas por *Helicobacter pylori* e da rinite alérgica e a prevenção da dermatite alérgica, havendo além disso outros possíveis efeitos benéficos ainda em estudo.

Para conferirem um efeito benéfico, os microrganismos probióticos atuam através de determinados mecanismos de ação. Vários destes mecanismos já foram descritos: 1) interação com o hospedeiro, através do fortalecimento da barreira intestinal, estimulação da produção de péptidos antimicrobianos, e interação com células do sistema imunitário, entre outros; 2) interação com outros microrganismos, como por exemplo competição por nutrientes limitantes, competição pela adesão ao trato gastrointestinal, ou atividade antimicrobiana contra estes; 3) interação com moléculas presentes no ambiente gastrointestinal.

O estudo das características e benefícios dos probióticos normalmente é realizado através de estudos *in vitro*, mas um método importante para a ligação destes com os seus efeitos em seres humanos é através da utilização de modelos animais *in vivo*. O nematode *Caenorhabditis elegans* é um modelo animal amplamente estudado, apresentando várias vantagens como a sua facilidade de manutenção e o seu ciclo de vida curto. Este nematode tem sido utilizado recentemente para o estudo dos efeitos probióticos de microrganismos, e pode também ser aplicado como um modelo de infeção para várias bactérias patogénicas.

O presente trabalho teve como objetivo o isolamento e caracterização de microrganismos probióticos a partir de fermentações de chucrute. Seis tipos de fermentações foram realizadas em triplicado, usando como substrato três variedades de couve (portuguesa, coração de boi ou lombarda) com ou sem a adição de ervas aromáticas. O pH, produção de ácido e quantidade de bactérias lácticas viáveis foram analisados ao longo do tempo das fermentações, procedendo-se também ao isolamento de bactérias lácticas durante este período. De seguida, os isolados foram caracterizados fenotipicamente, através da realização da coloração de *Gram* e dos testes da catalase e oxidase; e genotipicamente através de *PCR-fingerprinting* e *multiplex PCR*, permitindo a sua identificação ao nível do género. Isolados representativos foram avaliados em termos de segurança, através da determinação da atividade hemolítica e do perfil de resistência a compostos antimicrobianos; e do seu potencial probiótico, através da caracterização de atividade antimicrobiana e resistência a pH baixo e à presença de bílis. Para além disso, *C. elegans* foi utilizado como modelo animal para analisar o potencial probiótico ou patogénico dos isolados mais promissores.

As fermentações de chucrute parecem ter decorrido adequadamente, apresentando uma evolução dos seus parâmetros ao longo do tempo semelhante à descrita na literatura, apesar de alguns destes não atingirem os valores descritos. Quatro destas fermentações foram escolhidas e 114 isolados foram caracterizados através da realização de testes fenotípicos e genotípicos, que permitiram a escolha de isolados representativos. A maioria destes foram identificados como *Lactobacillus* spp. (52%), e alguns como *Leuconostoc* spp. (33%), sendo que a frequência de microrganismos destes géneros foi diferente entre as fermentações, claramente dependendo do tipo de couve utilizada como substrato. De igual modo, a diversidade microbiana também diferiu conforme a fermentação.

Em relação ao perfil de segurança, apenas um isolado apresentou atividade beta-hemolítica, e desta forma foi possível concluir que os isolados parecem possuir um reduzido grau de virulência. Relativamente à resistência a compostos antimicrobianos, os isolados apresentaram uma elevada percentagem de resistência à vancomicina, e uma baixa percentagem de resistência à ampicilina, cloranfenicol e clindamicina. A incidência de resistência à eritromicina, gentamicina, canamicina, estreptomicina e tetraciclina foi variável, dependendo do género em estudo. Em geral, 42% dos isolados mostraram resistência a pelo menos um composto antimicrobiano associado à transmissibilidade a outros microrganismos.

Quanto à resistência a condições que simulam o trato gastrointestinal, 88% dos isolados apresentaram resistência à presença de bílis, enquanto 20% demonstraram resistência a valores baixos de pH.

Seis isolados foram escolhidos com base nos resultados obtidos nos testes anteriores e mostraram atividade antimicrobiana contra *Listeria monocytogenes*. Três apresentaram resistência a valores mais baixos de pH do que os valores anteriormente testados.

Por fim, dois dos três microrganismos com maior potencial probiótico foram estudados utilizando o modelo *C. elegans*. Os isolados, por si só, não demonstraram nenhum efeito benéfico para o organismo dos nematodes. No entanto, quando os vermes foram colocados em contacto com uma estirpe patogénica de *Serratia* sp. após contacto com os candidatos probióticos, verificou-se um efeito sinergístico entre estes, ocorrendo desta forma o aumento da patogenicidade da estirpe de *Serratia* em estudo. Os resultados obtidos indicam que os microrganismos estudados podem não ser apropriados para aplicação como probióticos, e devem ser melhor caracterizados.

Em conclusão, 114 isolados de fermentações de chucrute foram caracterizados quanto ao seu potencial para aplicação como microrganismos probióticos, tendo-se verificado que três destes apresentaram características apropriadas para tal. Desta forma, comprova-se a aplicabilidade das fermentações de chucrute como fonte para o isolamento de microrganismos probióticos. Apesar de dois dos três candidatos não terem apresentado um efeito benéfico quando testados no modelo *C. elegans*, é importante referir que mais testes deverão ser realizados no futuro para avaliar o potencial probiótico destes microrganismos, nomeadamente através do uso de outras estirpes patogénicas ou da aplicação de outros modelos *in vivo*.

Palavras-chave: probióticos; bactérias lácticas; chucrute; *Caenorhabditis elegans*

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List of relevant abbreviations

AAD	Antibiotic-associated diarrhea
BHI	Brain heart infusion
CD	Crohn's disease
CFCS	Cell-free culture supernatants
GI	Gastrointestinal
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
LAB	Lactic acid bacteria
LB	Luria Broth
LGG	*L. rhamnosus* GG
MRS	de Man, Rogosa and Sharpe
NEC	Necrotizing enterocolitis
NGM	Nematode growth medium
RTI	Respiratory tract infections
UC	Ulcerative colitis

1 - Introduction

1.1 - Intestinal microbiota

The human body contains up to 10^{14} microbial cells, composing about 90% of all cells present. These microorganisms colonize various parts of the human body, with microbial diversity varying according to colonization site; however, the most colonized organ is the gastrointestinal (GI) tract. The community of microorganisms present there, either by transient or permanent colonization, is referred as the intestinal microbiota (Gerritsen *et al.*, 2011; Pflughoeft & Versalovic, 2012).

This microbiota influences the host at metabolic, nutritional, physiological and immunological levels. It extracts energy from dietary compounds that are not readily digested and produces important nutrients such as vitamins, amino acids and short-chain fatty acids, which are vital to humans. The microbiota also helps to develop and maintain gut and immune homeostasis, interacting with the intestinal barrier and the mucosal immune system. Furthermore, it is also involved in the defense against pathogens by production of antimicrobial compounds or by colonization impairment (Derrien & Van Hylckama Vlieg, 2015; Gerritsen *et al.*, 2011). Besides the interaction and effects within the GI tract, this microbiota also influences several other organs, like the nervous system and the brain, the liver or the cardiovascular system (Derrien & Van Hylckama Vlieg, 2015; Sekirov *et al.*, 2010).

The microbiota is composed of various types of microorganisms, such as archaea, viruses, protozoa and fungi, but bacteria are the most abundant and studied type of microorganism present in the GI tract (He *et al.*, 2013). Most bacteria present belong to two phyla, *Bacteroidetes* and *Firmicutes*, with other phyla, such as *Proteobacteria* and *Actinobacteria*, also being present in smaller proportions (Sekirov *et al.*, 2010).

More than 1000 species can be found in the GI tract of the total human population, but the actual number of species present in each individual is lower, reaching approximately 160 (Gerritsen *et al.*, 2011). The intestinal microbiota is therefore highly diverse among individuals, with only about half of the species present in an individual being shared with the vast majority of the human population. On the other hand, there are a number of species that are rare and variable between individuals. This indicates that there is a core and a variable microbiota, which are probably specific and stable within each person (Derrien & Van Hylckama Vlieg, 2015). Adding to this, the microbiota can also include a transient community, depending on variations in diet or in environmental factors (Derrien & Van Hylckama Vlieg, 2015).

Gut colonization begins directly at birth, when the newborn is exposed to the mother's microbiota and the surrounding environment. This colonization differs among newborns and depends on various factors (Gerritsen *et al.*, 2011). During the first year of life, intestinal microbiota complexity and diversity increases, with some fluctuations being observed, and by the

end of this period the microbiota present stabilizes, reaching a composition similar to that of adult humans (Pflughoeft & Versalovic, 2012; Sekirov *et al.*, 2010).

The composition of the intestinal microbiota of a healthy human depends on several factors, including host genetics, age and environmental factors, particularly diet and ongoing therapeutics, with the latter two playing an important role (Derrien & Van Hylckama Vlieg, 2015). Diet seems to be a major factor determining the microbiota of different individuals. Populations that have major divergences in diet present distinct proportions of dominant bacterial phyla and can present even functional changes, such as the presence of microorganisms that can digest seaweed on the microbiota of Japanese populations (Alcock *et al.*, 2014; Pflughoeft & Versalovic, 2012). Furthermore, short-term changes in an individual's diet can also induce alterations in the microbiota and in its total genome content, the microbiome, and these can occur quickly, sometimes within a day (He *et al.*, 2013). Oral administration of antibiotics also has a profound and disruptive effect in microbiota, with about 33% of the gut microbiome being suppressed after a five-day treatment with one antibiotic. Although the microbial community seems to be generally restored within weeks after treatment, several groups may be lost (Pflughoeft & Versalovic, 2012).

Perturbation of the normal microbiota of an individual, or dysbiosis, is associated with several diseases, though it is not generally known if it happens due to the disease or if it is the cause of its onset (Gerritsen *et al.*, 2011). Obesity seems to be affected by the microbiota present, as studies showed that obese and thin mice present differences in the composition of their gut microbial communities, and also that the microbiota of obese human twins is less diverse than the one from their thin siblings. In fact, transplantation of microbiota from slim mice normalized the weight of obese mice, and inoculation of germ-free mice with the microbiota from an obese human led to obesity (Alcock *et al*., 2014; Pflughoeft & Versalovic, 2012).

Dysbiosis is associated with diseases such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), with specific forms of IBD and IBS having different microbiota compositions, distinguishable from each other and from healthy individuals (Gerritsen *et al.*, 2011). The development of acute gastroenteritis is also connected with dysbiosis and reduced diversity of the microbiota, with this disturbance creating opportunities for pathogen proliferation and disease establishment. This mechanism can also be one of the causes of antibiotic associated diarrhea, due to the before mentioned disruptive effect of antibiotics (Pflughoeft & Versalovic, 2012). Other conditions linked with dysbiosis include type two diabetes, celiac disease, colorectal cancer, pouchitis and necrotizing enterocolitis (Gerritsen *et al.*, 2011).

Due to the importance of GI microbiota and its impact on human health, it is important for individuals to be able to modulate it. An important approach may be through the use of probiotics (Butel, 2014).

1.2 - Probiotics

Probiotics are defined by the Food and Agriculture Organization and the World Health Organization as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). The concept of probiotics was first introduced in the early 20th century, through the work of Nobel laureate Ilya Metchnikoff, who associated the longevity of Bulgarian farmers with their diet based on the consumption of fermented milk products. He then suggested that these products, specifically yoghurt, contained microorganisms that act by protecting the intestine from harmful bacteria (Parvez *et al.*, 2006). The first clinical trials regarding probiotics were performed in the 1930s, and research in the subject has greatly increased since then, with the definition of probiotics evolving to its present-day form (Parvez *et al.*, 2006; Vasiljevic & Shah, 2008). There is currently an increased interest in and demand for probiotics, in face of their recorded safe use and increasingly recognized effects on human health (Fontana *et al.*, 2013).

The majority of probiotic microorganisms belong to the lactic acid bacteria (LAB) group, which is composed of several genera, with the most important ones with probiotic strains being *Lactobacillus* and *Bifidobacterium*, but also *Leuconostoc*, *Streptococcus* or *Enterococcus* (Butel, 2014; Fontana *et al.*, 2013). LAB are Gram-positive, oxidase-negative, fastidious and strictly fermentative microorganisms, and the main product of their fermentation pathway is lactic acid (Vasiljevic & Shah, 2008). Other bacteria such as *Escherichia coli* or even yeast like *Saccharomyces boulardii* may also be used as probiotics (Butel, 2014).

1.2.1 - Probiotic products and sources

Probiotics may be included in foods or be marketed as a supplement, in the form of tablets, capsules or freeze-dried preparations. These food products may be the result of fermentation by probiotic microorganisms or probiotic-fortified products (Varankovich *et al.*, 2015). Fermented dairy products, especially yogurt, are the traditional probiotic foods, but recently other food matrices have been studied for their application as probiotic vehicles, such as vegetables, fruit juices (either fresh or fermented), cereal based products or drinks and fermented sausages (Rivera-Espinoza & Gallardo-Navarro, 2010). There is a growing interest in the development of alternatives to dairy based probiotic products, mainly due to their lactose and cholesterol content, and some non-dairy based probiotic products are already commercially available, such as fruit juices and oat based products (Martins *et al.*, 2013; Vasiljevic & Shah, 2008).

The number and viability of microorganisms present in the probiotic product is important to guarantee that the probiotic is consumed alive and in adequate quantities (Vasiljevic & Shah, 2008). It is generally accepted that probiotics must maintain viability in order to exert their beneficial effects, despite evidence that dead microbial cells can also present some of these health benefits, particularly those related with immunomodulation (Adams, 2010). Therefore, the characteristics of the product or food matrix used for the delivery of probiotics are important, since

they affect the viability of probiotic microorganisms. Factors that affect viability include: chemical composition, such as nutrient availability, pH and oxygen presence (particularly for anaerobic microorganisms); inoculum quantity; presence of and interaction with other microorganisms; fermentation time in the case of fermented products; and storage conditions, mainly temperature, and duration (Rivera-Espinoza & Gallardo-Navarro, 2010; Vasiljevic & Shah, 2008).

The two major sources of probiotic microorganisms are the human GI tract and fermented dairy products. Many bacteria have been isolated from human fecal samples, since GI tract strains are probably more adapted and have better survivability when passing through it (Rivera-Espinoza & Gallardo-Navarro, 2010). Dairy products, particularly fermented milks, have a complex composition in LAB species, representing another main source of probiotics (Fontana *et al.*, 2013). Probiotics can also be isolated from other substrates, with fermented non-dairy foods representing an important alternative source, particularly plant based products like fermented vegetables. Strains isolated from these products may be especially useful for application in probiotic products with similar origin, since bacteria from other sources might not possess acceptable viability traits to survive in these matrices (Peres *et al.*, 2012).

Sauerkraut is one of these vegetable products, resulting from the spontaneous fermentation of cabbage in anaerobic conditions after the addition of salt (Harris, 1998). The fermentation occurs over the course of several weeks, in temperatures ranging from 15 to 20°C, being performed by a microbial succession. This succession is characterized by two phases: a heterofermentative phase dominated by *Leuconostoc mesenteroides* followed by a homofermentative phase dominated by *Lactobacillus plantarum* (Holzapfel *et al.*, 2008; Plengvidhya *et al.*, 2007). The presence of salt and anaerobic conditions will favor the growth of heterofermentative microorganisms, particularly *Leuconostoc mesenteroides*, which is the most common LAB species in cabbage. These microorganisms promote the acidification of the product and create a more favorable environment to the homofermentative microorganisms, which will carry the second fermentation phase (Holzapfel *et al.*, 2008). While *Leuconostoc mesenteroides* and *Lactobacillus plantarum* are the predominant species in sauerkraut fermentation, there are other microorganisms present in lower numbers which may be important, mainly other species of *Leuconostoc* and *Lactobacillus*, as well as *Pediococcus* and *Weissella* (Plengvidhya *et al.*, 2007).

Although there are not many studies on the probiotic characteristics of sauerkraut microbiota, there have been reports of potentially probiotic strains isolated from this product (Beganović *et al.*, 2014; Peres *et al.*, 2012), as well as from related products such as kimchi and Chinese sauerkraut (Chang *et al.*, 2010; Yu *et al.*, 2013), which underlines that sauerkraut may be an important source of probiotic microorganisms.

1.2.2 - Characteristics of probiotic microorganisms

The selection of a microorganism as a probiotic depends on several required characteristics. These can vary highly between members of the same genus and even species,

and therefore it is important to first identify the species and then characterize the strain (Aureli *et al.*, 2011).

1.2.2.1 - Safety characteristics

A microorganism must be safe for human ingestion before it can be used as a probiotic. Probiotics have a good history of safe application, but there have been some rare cases of harmful effects associated with their ingestion. There have been reports of infections, including endocarditis (Mackay *et al.*, 1999), caused by *Lactobacillus* species, and some cases of fungemia by *S. boulardii* (Boyle *et al.*, 2006), but all reports of probiotic sepsis are associated to either chronic disease, debilitation or immunocompromised patients (Boyle *et al.*, 2006). A single clinical trial has shown that increased mortality is associated with probiotic use in patients with acute pancreatitis, with this being the only study found that makes such an association in a clinical setting (Gareau *et al.*, 2010).

Another safety issue is the possibility that probiotics may serve as a reservoir for antibiotic resistance genes. Antibiotic resistance can be classified as intrinsic or acquired. The first is present in all members of a certain species or genus and is considered non-transmissible between microorganisms, while the latter is present in only some strains of the group and is acquired either by random chromosomal mutations (which have a small risk of transmission) or by horizontal gene transfer (Clementi & Aquilanti, 2011). Antibiotic resistance genes are often acquired through plasmids or transposons, being themselves easily transmitted to other microorganisms (Varankovich *et al.*, 2015). This is an important aspect, since probiotics that have these transferrable genes can act as reservoirs and help spread antibiotic resistance to pathogenic microorganisms (Devirgiliis *et al.*, 2013).

Generally, microorganisms to be used in the food industry in Europe must follow a "Qualified Presumption of Safety" regulation, which establishes groups of microorganisms that are considered safe for consumption. Microbial strains identified and classified as belonging to these groups only have to be tested for the absence of virulence factors, such as hemolytic activity, and antibiotic resistance (Aureli *et al.*, 2011).

1.2.2.2 - Functional characteristics

A probiotic strain must possess several properties in order to function as a probiotic and produce health benefits to the host, with the most important characteristics being resistance to GI conditions and adherence to the GI tract.

One of the most important characteristics needed is the ability to survive transit through the GI tract, in order to maintain viability and colonize the human host (Papadimitriou *et al.*, 2015). Probiotics are subjected to different stresses along the GI tract, with the first stressful conditions being present in the stomach, where they must tolerate low pH (which fluctuates between 1-2 and 4-5) and the action of digestive enzymes like pepsin (Derrien & Van Hylckama Vlieg, 2015; Papadimitriou *et al.*, 2015). After leaving the stomach, microorganisms come into contact with the

small intestine environment, which has a higher, less stressful pH (>6) but contains several enzymes or aggressive compounds, such as bile, pancreatin and lipase (Derrien & Van Hylckama Vlieg, 2015). Tests used for the assessment of survival through the GI tract include the use of buffers or traditional media adjusted to low pH or containing bile, which allow screening of a large number of strains, the use of artificial gastric and pancreatic juices, and even *in vitro* simulators of the GI tract (Papadimitriou *et al.*, 2015).

Adherence to the GI tract, either to the epithelial cells or the mucus layer, is generally regarded as a beneficial characteristic and is important for helping host colonization and pathogen exclusion (Fontana *et al.*, 2013). Nonetheless it can also be considered as a risk factor that can increase translocation and invasion of the GI tract by the probiotic strain, which may be important when the probiotic consumers are immunocompromised (Papadimitriou *et al.*, 2015).

There are several tests available to assess this ability, such as auto-aggregation assays, tests based on adherence to intestinal mucus, intestinal epithelial cell lines, intestinal tissue fragments or even models consisting of epithelial tissue, mucus and the commensal microbiota (Papadimitriou *et al.*, 2015).

1.2.3 - Benefits for the human health

There are several benefits that are proved to be associated with probiotic ingestion, particularly related to the treatment of GI diseases. In addition, other benefits on the human health are thought to be caused by probiotics, but need to be further studied in order to be established as such. A summary of these benefits is presented on **Table 1**. Like the other characteristics mentioned, health benefits are strain specific, with proven effects only being applicable to the strain or group of strains tested, and while some can provide more than one benefit, there is no universal strain that can provide all of the proposed benefits (Vasiljevic & Shah, 2008). Furthermore, responses to probiotics may differ between each individual host and may depend on their health status (Bron *et al.*, 2011).

1.2.3.1 - Gastrointestinal diseases

As previously mentioned, antibiotic treatment can cause dysbiosis of gut microbiota, and one of the most common side-effects of antibiotic treatment is antibiotic-associated diarrhea (AAD), with an incidence of about 5 to 39%, depending on the type of antibiotic (Varankovich *et al.*, 2015). While there may be several causes for AAD, *Clostridium difficile* is the primary etiologic agent of this disease, accounting for about 15-25% of the cases (Pflughoeft & Versalovic, 2012). In this case, disruption of the microbiota may provide a chance for the opportunistic pathogen to proliferate, which leads to the production of toxins, causing the disease (Varankovich *et al.*, 2015). *Cl. difficile* AAD is one of the most common nosocomial infections and can lead to severe complications, being a frequent cause of morbidity and mortality among elderly hospitalized patients (Varankovich *et al.*, 2015).

One of the most important established beneficial effect of probiotics is their application for preventing and treating AAD. Many studies have supported the use of probiotics for diarrhea treatment, with a literature meta-analysis finding 82 studies that together provide evidence of the effect of probiotics in the treatment of this condition, based on microorganisms of several genera (Hempel *et al.*, 2012). *Saccharomyces boulardii* appears to be the most effective microorganism for AAD prevention, but *Lactobacillus rhamnosus* GG is also proven to be effective in children (Aureli *et al.*, 2011). For *Cl. difficile* AAD, there is also evidence for the use of probiotics in aiding prevention and treatment, with *S. boulardii* being effective in the prevention of disease reoccurrence (Gareau *et al.*, 2010). Nonetheless, large multi-center studies should be performed to acquire more evidence for the use of probiotics in the context of *Cl. difficile* infection (Varankovich *et al.*, 2015).

Infectious gastroenteritis includes several diseases caused by different types of pathogens, which can either be viruses, bacteria or protozoa, and commonly occurs after the consumption of contaminated food or water. Viral pathogens are more predominant in young children, while children older than three years of age are typically more prone to infection with bacterial pathogens (Gareau *et al.*, 2010; Pflughoeft & Versalovic, 2012). There is evidence that probiotics have an effect in the treatment of infectious gastroenteritis, particularly in the pediatric population. The most effective probiotic seems to be *L. rhamnosus* GG, with studies showing that treatment reduced the duration of symptoms and their frequency (Pieścik-Lech *et al.*, 2013). Studies have confirmed the positive effect of *S. boulardii* in the treatment of the disease, and also of other organisms, such as various strains of *Lactobacillus reuteri*, *Bifidobacterium animalis* Bb-12 and *Lactobacillus paracasei* ST11 (Aureli *et al.*, 2011; Pieścik-Lech *et al.*, 2013; Vasiljevic & Shah, 2008).

Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in premature infants, affecting approximately 7% of very low birth weight infants, with a mortality rate of 20-30%. The etiology of this disease is not completely understood, but several risk factors are thought to be involved, such as prematurity, formula feeding, altered microbiota, neonatal stress and prolonged antibiotic treatment (Gareau *et al.*, 2010; Patel & Denning, 2015). Studies show that probiotic use is effective for the treatment and prevention of NEC, significantly reducing its incidence and mortality while not having negative effects in children growth and development (Gareau *et al.*, 2010). Furthermore, it seems that different subgroups of probiotics such as strains of *Lactobacillus*, *Bifidobacterium* and *Saccharomyces* genera are equally effective in the treatment of NEC (Patel & Denning, 2015).

Inflammatory bowel disease (IBD) is the designation for several inflammatory disorders of the GI tract, characterized by altered gut permeability, inflammation and ulceration (Gareau *et al.*, 2010). The most frequent forms are Crohn's disease (CD) and ulcerative colitis (UC), which are both chronic and relapsing diseases. CD consists of discontinuous inflammation of the whole GI tract and deep ulcers while UC is restricted to the colon and rectum and consists of continuous inflammation and superficial ulcers (Gerritsen *et al.*, 2011). Some patients with UC must undergo colectomy with ileal pouch-anal anastomosis, which creates an ileal pouch. Inflammation of this

pouch is known as pouchitis and is another form of IBD (Isaacs & Herfarth, 2008). The underlying causes of IBD are still not completely understood, but genetic factors and microbiota dysbiosis have been associated with the disease, and it is thought that aberrant host response to the gut microbiota might be involved (Pflughoeft & Versalovic, 2012; Sekirov *et al.*, 2010). The effect of probiotics in IBD varies in effectiveness according to the form of disease. Probiotic treatment seems to be effective in maintaining UC remission, and beneficial effects have been shown for *E. coli* Nissle 1917 and VSL#3, which is a combination of four *Lactobacillus* spp., three *Bifidobacterium* spp. and *Streptococcus thermophilus* (Fontana *et al.*, 2013; Hart & Hendy, 2014; Wohlgemuth *et al.*, 2010). VSL#3 is also effective in the treatment of pouchitis, reducing reoccurrence of disease, with about 85% of patients treated with the probiotic maintaining remission after one year (Fontana *et al.*, 2013). On the other hand, several studies have found that probiotic treatment seems to have no effect on CD (Fontana *et al.*, 2013).

Irritable Bowel Syndrome (IBS) is a chronic condition affecting 5 to 20% of the population, and is characterized by abdominal pain, bloating and altered bowel movements (Ford *et al.*, 2014; Gerritsen *et al.*, 2011). There are three subtypes of disease, depending on the type of bowel movement dysfunction: diarrhea-predominant IBS, constipation-predominant IBS, or an alternation between the two (Gerritsen *et al.*, 2011). The causes of IBS are not yet clearly understood, but several factors have been implicated, including dysbiosis of intestinal microbiota (Ford *et al.*, 2014). Probiotic use has a beneficial effect in the treatment of IBS, both in children and adults, and is associated with improvement in global IBS symptoms and reduced abdominal pain (Fontana *et al.*, 2013). However, the quality of some of the clinical trials performed in this area has been questioned, and better designed studies must be performed (Santos & Whorwell, 2014). Various strains, both from *Lactobacillus* and *Bifidobacterium* genera, and strain combinations, help alleviate individual IBS symptoms, with some showing more promising results, as is the case of *Bifidobacterium infantis* 35623, which seems to improve all symptoms significantly (Gareau *et al.*, 2010; Quigley, 2010). The best probiotic for IBS has not yet been defined, but it is thought that different probiotics may be ideal for the treatment of different IBS subgroups (Gareau *et al.*, 2010; Santos & Whorwell, 2014).

Probiotics have also been investigated for application in the treatment of *Helicobacter pylori* infection, which in the long term may lead to chronic gastritis, peptic ulcers and increased risk of cancer in the GI tract. Probiotic microorganisms show promising results in the treatment of this infection, particularly *S. boulardii*, as an adjuvant to other therapies (Malfertheiner *et al.*, 2012; Parvez *et al.*, 2006).

1.2.3.2 - Other beneficial effects

Probiotics have also been studied in the context of allergic diseases such as asthma, allergic rhinitis, atopic dermatitis and atopic eczema. These diseases are characterized by elevated serum immunoglobulin E levels, which are caused by an imbalance between immune system T helper 1 and T helper 2 cells, with a shift for the later type (Adams, 2010). Studies have shown that the use of probiotics help in the treatment of allergic rhinitis and have an effect in the

prevention of pediatric atopic dermatitis. However, there is no evidence for the beneficial effect in the treatment of asthma and atopic eczema (Fontana *et al.*, 2013). Interestingly, heat-killed probiotic cells seem to improve T helper cell balance and inhibit immunoglobulin E production *in vitro* and in animal models, which indicates that viability may not be a requirement for these effects (Adams, 2010).

There is evidence that probiotics help to prevent and to reduce the severity of respiratory tract infections (RTI). A meta-analysis reported that probiotic treatment may help prevent acute upper RTI, and reduce antibiotic administration (Fontana *et al.*, 2013). Results indicate that probiotics reduce the risk of recurrent respiratory infections during the first year of life, while in adults they reduced the severity and duration of RTI, but did not help their prevention (Aureli *et al.*, 2011; Rautava *et al.*, 2009). In elders, a probiotic product was shown to reduce the duration of RTI, especially upper RTI and nasopharyngitis (Aureli *et al.*, 2011).

The role of probiotics in the prevention of cancer, particularly colorectal cancer, has been investigated. Although there is a lack of data linking probiotics and the risk of colorectal cancer in humans, *in vitro* and *in vivo* studies have provided evidence for some mechanisms of action of probiotics which can lead to prevention of colorectal cancer risk (Chong, 2014). Furthermore, several studies have found that preparations of LAB inhibit the proliferation of tumor cells in animals (Vasiljevic & Shah, 2008).

Other proposed beneficial effects of probiotics include: improvement of celiac disease (de Sousa Moraes *et al.*, 2014), weight loss (Alcock *et al.*, 2014) and improvement of mood and reduction of anxiety (Dinan *et al.*, 2013).

Table 1 - Main health benefits of probiotic microorganisms. The major proven health benefits are the treatment of GI diseases, while for other diseases there are promising benefits that need to be confirmed.

Health Benefit	Disease	Effects
Proven	**AAD and *Cl. difficile* AAD**	Prevention, treatment or prevention of reoccurrence (Gareau *et al.*, 2010; Varankovich *et al.*, 2015)
	Infectious gastroenteritis	Reduced duration, severity of symptoms and risk, particularly in children (Pieścik-Lech *et al.*, 2013)
	NEC	Treatment and prevention of disease (Gareau *et al.*, 2010)
	IBD (UC and pouchitis)	Reduced reoccurrence (Fontana *et al.*, 2013; Hart & Hendy, 2014)
	IBS	Improvement of symptoms and abdominal pain (Fontana *et al.*, 2013; Gareau *et al.*, 2010)
	RTI	Prevention and/or reduction of severity and duration (Aureli *et al.*, 2011; Fontana *et al.*, 2013)
Promising	***H. pylori* infection**	Potential in pathogen eradication or as an adjuvant to other treatments (Malfertheiner *et al.*, 2012)
	Allergic rhinitis	Treatment adjuvant (Fontana *et al.*, 2013)
	Atopic dermatitis	Potential effect in prevention (Fontana *et al.*, 2013)

AAD - antibiotic-associated diarrhea; NEC - necrotizing enterocolitis; IBD - inflammatory bowel disease; UC - ulcerative colitis; IBS - irritable bowel syndrome; RTI - respiratory tract infections.

1.2.4 - Mechanisms of action

Probiotics may exert their beneficial effects through several different proposed mechanisms (**Figure 1**), which can be categorized into three types: i) interaction with the host; ii) interaction with other microorganisms; iii) interaction with molecules in the gut. Similarly to health benefits, different strains may have different mechanisms of action (Sherman *et al.*, 2009).

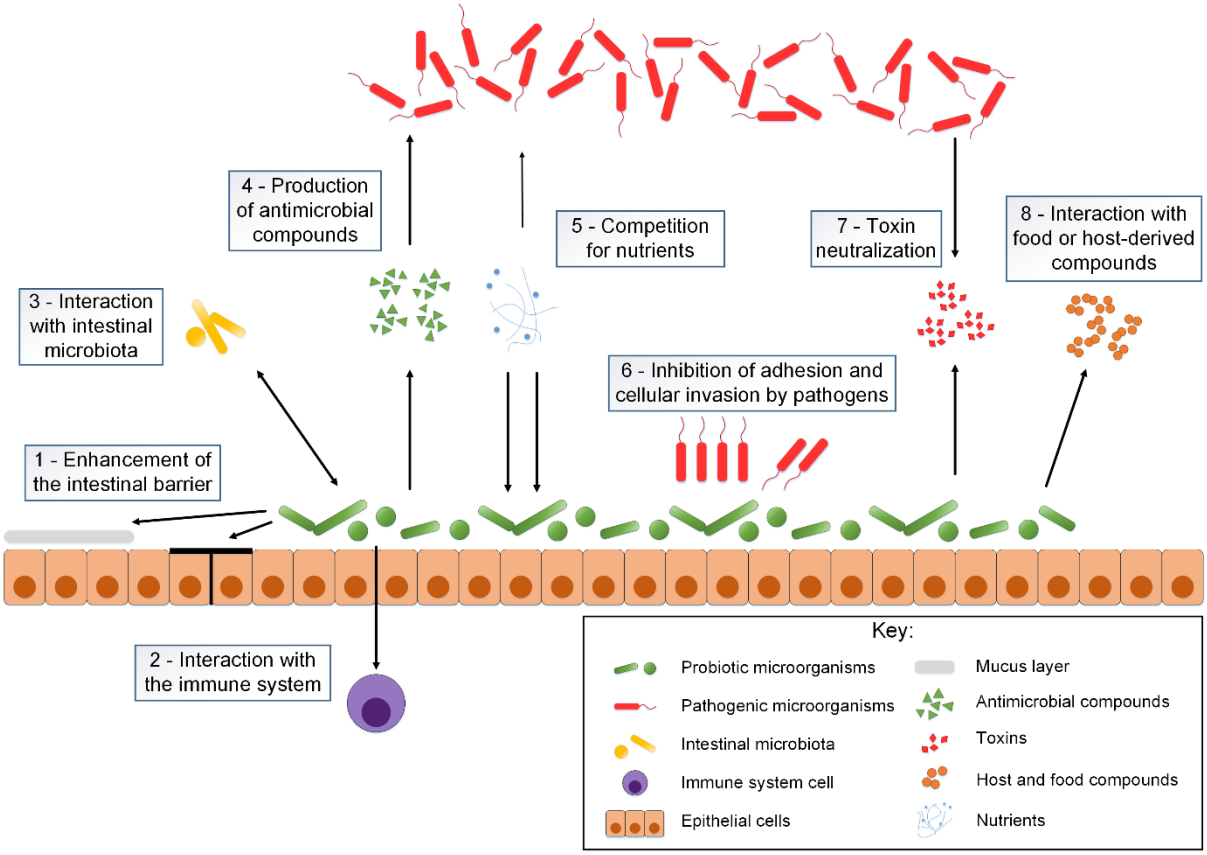

Figure 1 - Mechanisms of action of probiotic microorganisms: interaction with the host (1 - enhancement of the intestinal barrier, either by strengthening the epithelial barrier or by production of mucus; 2 - interaction with the immune system, favoring either pro or anti-inflammatory responses); interaction with microorganisms (3 - direct or indirect interaction with intestinal microbiota; 4 - production of antimicrobial compounds; 5 - competition for limited nutrients; 6 - inhibition of adhesion and cellular invasion by pathogens; 7 - toxin neutralization and inhibition); 8 - interaction with compounds present in the intestinal environment (such as mutagenic compounds). Figure based on information from Oelschlaeger, 2010, Sassone-Corsi & Raffatellu, 2015 and Wohlgemuth *et al.*, 2010.

1.2.4.1 - Interaction with the host

The intestinal barrier is an important defense mechanism of the human body. Its disruption can lead to pathogen invasion or to induction of inflammatory responses by bacterial and food antigens (Wohlgemuth *et al.*, 2010). The first barrier against adhesion and invasion by

pathogenic microorganisms is the mucus layer, which covers the intestinal epithelium and is composed of large glycoproteins, mucins (Sassone-Corsi & Raffatellu, 2015). Several probiotic strains have been shown to increase mucin gene expression and secretion in cell cultures or in animal models such as rats (Chong, 2014; Derrien & Van Hylckama Vlieg, 2015).

Tight junctions between intestinal cells are another barrier against pathogen invasion, by helping to conserve the structural integrity of the epithelial layer and controlling the extracellular permeability of this tissue (Chong, 2014). Probiotics such as *E. coli* Nissle 1917, *Lactobacillus casei* DN-114-001 and VSL#3 can help to maintain and repair the structure of these tight junctions, through increased expression or redistribution of their proteins (Bron *et al.*, 2011; Wohlgemuth *et al.*, 2010).

Probiotics can additionally protect the intestinal barrier by inhibition of epithelial cell apoptosis, reduction of epithelial damage and promotion of cell growth. Two proteins secreted by *L. rhamnosus* GG have been shown to activate specific pathways that lead to these effects, and proteins with a similar role have been found in other probiotic strains (Bron *et al*., 2011; Wohlgemuth *et al.*, 2010).

The GI tract produces antimicrobial peptides as a way of strengthening the intestinal barrier and preventing infection, compounds which are secreted by the Paneth cells in the small intestine (Sassone-Corsi & Raffatellu, 2015). Recognition of specific components of the probiotic microorganisms, such as the cell wall or flagella, by the Paneth cells has been shown to enhance production of these peptides *in vitro* and *in vivo* (Oelschlaeger, 2010; Sassone-Corsi & Raffatellu, 2015).

Probiotics can also have an effect on other cells of the immune system, such as dendritic and T cells. These host cells can interact with microorganisms in the GI tract through recognition of molecular patterns by pattern recognition receptors, such as toll-like receptors, which activates complex cellular signal transduction cascades. This leads to either a pro-inflammatory or an anti-inflammatory immune response, each of these being associated with increased expression of different cytokines (Bron *et al.*, 2011; Wohlgemuth *et al.*, 2010). Probiotics *L. rhamnosus* GG, *L. reuteri*, *L. casei* and VSL#3 have been shown to increase interleukin-10 production (an anti-inflammatory cytokine) and/or reduce the production of several pro-inflammatory cytokines in this way (Wohlgemuth *et al.*, 2010).

Production of cytokines can also be stimulated by interaction with other cells, such as intestinal and colonic epithelial cells. For example, *L. rhamnosus* GG or soluble compounds secreted by *E. coli* Nissle 1917 and VSL#3 can cause decreased production of interleukin-8, a pro-inflammatory cytokine, by these cells (Marco *et al.*, 2006; Oelschlaeger, 2010). On the other hand, *E. coli* Nissle 1917 has been associated with the up-regulation of genes related with pro-inflammatory response in epithelial cell cultures (Wohlgemuth *et al.*, 2010).

Furthermore, some probiotic strains can promote the differentiation of B cells into plasma cells, and therefore increase the production of secretory immunoglobulin A. These antibodies bind to pathogenic microorganisms in the mucus layer, helping reduce the colonization of the intestinal epithelium (Sherman *et al.*, 2009).

1.2.4.2 - Interaction with other microorganisms

Probiotic microorganisms can interact with other microorganisms present in the GI tract, particularly pathogens, but also with the gut microbiota. The microorganisms may be influenced by the modification of the metabolic networks present in the gut, affecting its environment (Derrien & Van Hylckama Vlieg, 2015).

On the other hand, efficient competition for limiting nutrients such as carbohydrates and iron can lead to the inhibition of pathogenic microorganism multiplication. An example of this mechanism is shown by *E. coli* Nissle 1917, which can out-compete the pathogen *Salmonella* Typhimurium for the colonization of the inflamed intestine. This is due to the presence of several iron-acquisition systems in the probiotic strain, which allow it to better grow in this iron-limited environment (Sassone-Corsi & Raffatellu, 2015).

Furthermore, probiotics have also been shown to compete with pathogens for the adhesion to epithelial cells *in vitro*. This mechanism, also referred as competitive exclusion, is based on the binding of both types of microorganisms to the same receptors on the epithelial surface, and can prevent colonization by several pathogens, like *Cl. difficile*, *Listeria monocytogenes*, *Staphylococcus aureus* and certain *E. coli* strains (Wohlgemuth *et al.*, 2010).

Some probiotic strains can also specifically interfere with the invasion of host epithelial cells by pathogenic microorganisms. It has been shown that *E. coli* Nissle 1917 can protect epithelial cells *in vitro* from invasion by several pathogens, such as *Salmonella* Typhimurium, *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila* and *Listeria monocytogenes*. This effect is mediated by secreted compounds, requiring no direct contact with the probiotic cells. Likewise, secreted compounds from some *Lactobacillus* and *Bifidobacterium* strains can inhibit invasion by *Salmonella* Typhimurium (Oelschlaeger, 2010).

Probiotic microorganisms can also produce various antimicrobial compounds, such as organic acids, hydrogen peroxide and bacteriocins. Organic acids are the main products of LAB fermentation and their production decreases GI tract pH, which can inhibit microbial growth (Rivera-Espinoza & Gallardo-Navarro, 2010).

Bacteriocins are small heat-stable antimicrobial peptides. Most of these peptides have a narrow spectrum of action, having an effect against closely related bacteria, but some present a broad activity spectrum (Vasiljevic & Shah, 2008; Wohlgemuth *et al.*, 2010). It has been shown that *Lactobacillus salivarius* UCC118 can protect mice against infection by *Listeria monocytogenes* through the production of a bacteriocin, highlighting the importance of these compounds (Corr *et al.*, 2007).

Another mechanism of probiotic action is the inhibition of toxin production and/or toxin neutralization. Several probiotic strains are able to inhibit the expression of shiga toxin by *E. coli* O157:H7 due to the production of organic acids. Certain probiotics, such as *Lactobacillus rhamnosus*, are also able to protect against cyanobacterial toxins or mycotoxins, reducing their intestinal absorption and increasing fecal excretion due to toxin binding. Furthermore, *Saccharomyces boulardii* was shown to protect against *Cl. difficile* toxin A, resulting from

interference with toxin action, induction of a specific immunoglobulin A immune response and secretion of a protease against the toxin (Oelschlaeger, 2010).

1.2.4.3 - Other mechanisms

Probiotics are also thought to help in the prevention of cancer, through anti-carcinogenic effects. Some of these mechanisms already described include the modulation of the immune system and reinforcement of the intestinal barrier. However, other mechanisms have also been proposed to generate this effect, like antimutagenic activity against certain chemical compounds, binding of mutagenic compounds or interaction with bacterial enzymes that activate and produce carcinogenic compounds (Chong, 2014).

1.3 - *Caenorhabditis elegans* as a model for studying probiotics

An important method for studying probiotics is through *in vivo* studies, using animal models. Diverse models have been applied in probiotic research, from invertebrates such as nematodes and flies, to zebrafish and mammals like mice and rats, with each model presenting specific advantages and disadvantages (Papadimitriou *et al.*, 2015; Varankovich *et al.*, 2015).

Caenorhabditis elegans, a free-living nematode, is a well-known animal model. The worm feeds on bacteria, is able to grow between 12 and 25 ºC, and exists primarily as a hermaphrodite, capable of self-fertilization (Corsi *et al.*, 2015). *C. elegans* has a short life cycle (**Figure 2**), taking about three days to grow from the egg to an egg-laying adult. Eggs are laid early in embryo development and hatch into a L1 larva. Then the nematode goes through other development stages until it reaches its L4 stage (young adult). After about 12 h, the L4 nematode, now referred to as an adult, starts producing progeny, and will do so during approximately two to three days. After the reproductive period, it can survive for several more weeks before dying of senescence (Corsi *et al.*, 2015).

The use of *C. elegans* as a model for evaluating probiotic effects in the gut is an emerging field, and it has been applied to assess the anti-infective (Kim & Mylonakis, 2012), antioxidant, and lifespan extending (Grompone *et al.*, 2012) potential of probiotic strains. It has also been proposed for the study of anti-tumor activity of these strains (Clark & Hodgkin, 2014; Papadimitriou *et al.*, 2015). The nematode can be used as an infection model for several microorganisms, such as *Salmonella* Typhimurium, enteropathogenic *E. coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Serratia marcescens* (Marsh & May, 2012).

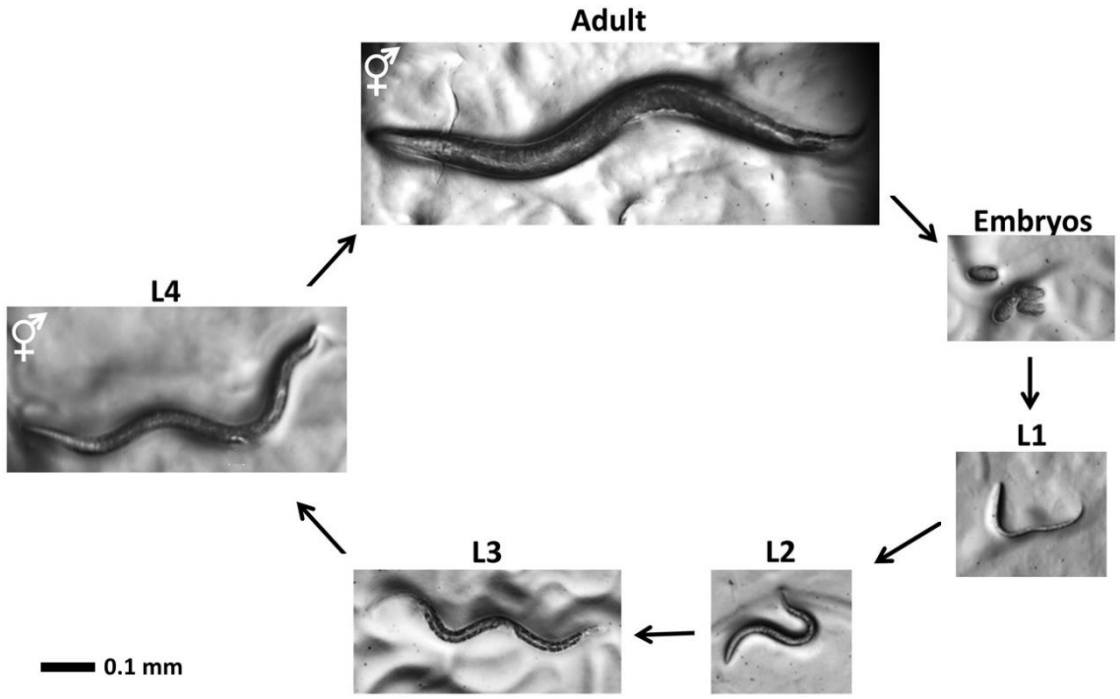

Figure 2 - Simplified life-cycle of *C. elegans*. The nematodes hatch from the eggs and undergo larval development until they reach the L4 stage. Adult individuals are L4 worms which are able to produce eggs. Adapted from Corsi *et al.*, 2015.

C. elegans presents several characteristics that make it useful as an animal model: it is easy and inexpensive to maintain in laboratory conditions; its short life cycle and large number of offspring permits a large-scale production of animals within short periods of time; its transparent body allows observation of internal organs of live individuals, such as the gut; and its intestinal cells are similar in structure to human intestinal cells (Clark & Hodgkin, 2014; Leung *et al.*, 2008; Park *et al.*, 2014). Nonetheless, it presents some disadvantages, such as a simpler immune system, and the absence of cellular invasion, an essential step in some pathogens' infection (Marsh & May, 2012).

1.4 – Aims of the study

Due to the relevance of probiotics to human health, it is important to find strains that provide health benefits or can be used in non-dairy probiotic products. Vegetable products may be an alternative source for the isolation of probiotics, with one of these products being sauerkraut. Various species of LAB can be found throughout the fermentation process of sauerkraut, and therefore it can be a potential source of probiotic candidates. There are several characteristics important for a microorganism to be used as a probiotic, with the most important being its safety, resistance to GI tract environmental conditions and ability to colonize and produce

a health effect on the host. These characteristics are usually studied by *in vitro* methods, followed by confirmation using an animal model.

Taking this into account, the main objective of the present work was to isolate LAB species from sauerkraut fermentations and select the strain(s) with the highest potential to be used as a probiotic microorganism. The following steps were performed in order to obtain and characterize the probiotic candidates:

1) Performance of sauerkraut fermentations and isolation of lactic acid bacteria;
2) Phenotypic and genotypic characterization of the isolates;
3) Safety evaluation and characterization of their probiotic potential;
4) Application of *C. elegans* as an animal model for the evaluation of toxicity and probiotic potential of selected isolates.

2 - Materials and methods

2.1 - Isolation of lactic acid bacteria from sauerkraut

Three types of biological cabbage were used as substrate for different sauerkraut fermentations: portuguese cabbage (also known as tronchuda cabbage, *Brassica oleracea* var. *costata*), pointed-head cabbage (*Brassica oleracea* var. *capitata*) and savoy cabbage (*Brassica oleracea* var. *sabauda*). Additionally, for each type of cabbage two recipes for sauerkraut production were performed, one using just salted cabbage as substrate, and the other using this substrate with added aromatic herbs and garlic (*Allium sativum*). Aromatic herbs used included lavender (*Lavandula* sp.), laurel (*Laurus nobilis*), rosemary (*Rosmarinus officinalis*) and thyme (*Thymus vulgaris*). Each cabbage/recipe fermentation was performed in triplicate, leading to a total of 18 fermentations.

Fermentations were performed as follows: after removing the outer leaves, cabbages were washed and sliced into thin strips (approximately 300 g of cabbage per assay); then, 3% w/w NaCl (Merck) was added, and the mixture was kneaded for a few minutes until its juices were released. For the aromatic herbs and garlic recipe, one garlic clove, one laurel leaf and one tablespoon of each other herb were minced and added before kneading. Each mixture was then uniformly distributed in six sterile plastic bottles, filled to the top with a 3% NaCl solution (final volume of 150 ml) and closed tightly. Fermentations were incubated at 20°C for 2, 5, 7, 16, 23 or 30 days. Before starting the fermentations and at each time-point, pH was measured and samples were taken from the sauerkraut and fermentation juice for LAB isolation and further microbial and acid quantification. A representation of the procedure followed for the production of sauerkraut is shown in **Figure 3.**

Production of acid was evaluated by titration of the fermentation juice with NaOH. For this purpose, 5 ml of fermentation juices were diluted with 5 ml of water, and two drops of phenolphthalein were added. This solution was then titrated with 0.1 N NaOH until its color changed. In the case of the initial, day 0, samples, 0.01 N NaOH was used instead due to the low quantity of acid present at the start of fermentation. The pH of the titrated solution was determined to confirm that the equivalence point had been reached and the titration had been successfully performed. Acidity was calculated as percentage of lactic acid, with the assumption that this was the primary acid produced during fermentation.

Quantification of LAB was performed as follows: 25 g of non-fermented salted cabbage or fermented sauerkraut and 225 ml of buffered peptone water (Oxoid, UK) were added to a stomacher bag, and the mixture was homogenized in a LabBlender 400 stomacher (Seward Limited, UK) for 90 s. Ten-fold serial dilutions in buffered peptone water were then performed from this suspension (10^{-1}) and were inoculated in de Man, Rogosa and Sharpe (MRS) agar (VWR, USA). For the initial salted cabbage samples the 10^{-1} dilution was inoculated, and for the fermented sauerkraut samples the three highest dilutions (initially the 10^{-4}, 10^{-5} and 10^{-6} dilutions, but varying according to results in earlier time-points) were inoculated. Plates were incubated at

37°C for 24-72 h, and colonies were counted in plates presenting 30 to 300 colonies. For each sample, the plate with the highest countable number of colonies was selected, and five representative colonies with the maximum diversity in terms of morphology were chosen for further purification. Colonies were purified by streaking at least four times in MRS agar, and conserved in buffered peptone water + 20% glycerol at -20°C.

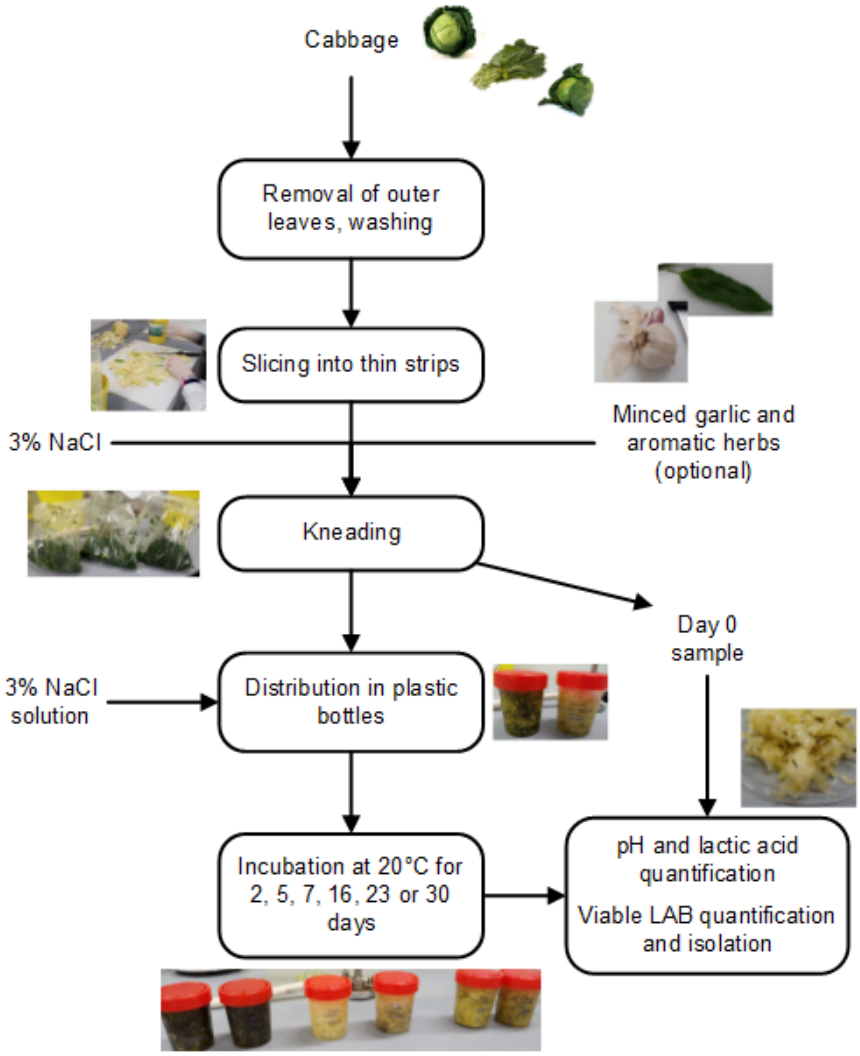

Figure 3 - Experimental procedure used for the preparation of sauerkraut fermentations.

2.2 - Phenotypic and genotypic characterization of the isolates

After isolation from sauerkraut, LAB isolates were first characterized phenotypically, by classic microbiological tests, and then genotypically, by PCR-fingerprinting in order to choose representative isolates and by multiplex-PCR for genus identification.

Phenotypic characterization was achieved by performing Gram stain, oxidase and catalase tests for the chosen isolates. Gram-positive, oxidase-negative and catalase-negative or weakly positive isolates were then submitted to genotypic analysis.

In order to perform the subsequent methods, DNA was extracted by the boiling method (Millar *et al.*, 2000). Briefly, a colony was suspended in 50 µl of Tris-EDTA with 0.1% Tween 20 (Merck), and incubated for 10 min at 100°C. Then, the suspension was centrifuged at 14000 rpm for 2 min using a Hermle® Z233 MK-2 centrifuge (Hermle, Germany), and the supernatant used directly in PCR reactions.

For fingerprinting analysis, six reaction mixtures were first tested using reference strains. The reaction mixtures differed only on the primer or primers used (StabVida, Portugal): BOXA1R; ERIC1R/ERIC2; $(GTG)_5$; M13; OPC-15; or REP1R/REP2 (**Table 2**). The reference LAB strains used were *Pediococcus pentosaceus* CECT 923, *Pediococcus acidilactici* DSM 20284^T, *Lactococcus lactis* CECT 5386, *Leuconostoc citreum* CECT 4025^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219^T, *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* CECT 748^T, *Lactobacillus coryniformis* subsp. *coryniformis* CECT 982^T, *Lactobacillus paracasei* Lb446R, *Lactobacillus curvatus* CECT 904^T and *Lactobacillus mali* M12. The two mixtures that presented the best results were chosen, and separate reactions were performed for the isolates under study using those primers.

PCR reactions were performed in a final volume of 20 µl, containing 10 µl of NZYTaq 2× Green Master Mix (NZYtech, Portugal), 50 pmol of primer and 1 µl of DNA. Amplification was performed using a Doppio thermocycler (VWR, USA) and the following conditions: 5 min at 95°C; followed by 40 cycles of 1 min at 95°C, 2 min at 40°C and 2 min at 72°C; and a final extension step of 10 min at 72°C, followed by refrigeration at 4°C.

After amplification, 2 µl of GelStar 10X (Lonza Rockland, USA) was added to 10 µl of PCR product and 10 µl of this mixture was submitted to electrophoresis in a 1.2% agarose gel (NZYtech, Portugal) with 0.5X Tris-Borate-EDTA (TBE) buffer (BioRad), using NZYDNA Ladder VIII (NZYtech, Portugal) as the DNA ladder. Agarose gels were visualized by transillumination under UV light using ImageMaster (Pharmacia Biotech, GE Healthcare, UK). The reproducibility of the technique was evaluated by performing 10% biological replicates, randomly selected from the isolates under study.

Fingerprinting profiles were analyzed using BioNumerics software, version 6.6.5 (Applied Maths, Belgium). Profiles were normalized and grouped using the Pearson correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA). Dendrograms were then produced, and representative LAB chosen for further testing.

LAB diversity of the fermentations was analyzed through the creation of separate dendrograms using the profiles from the isolates of each of the chosen fermentations. Diversity was assessed and compared by calculating the Simpson's diversity index (D'), using the following formula (Hunter & Gaston, 1988):

$$D' = 1 - \frac{1}{N(N-1)} \sum_{k=1}^{S} n_k(n_k - 1)$$

N - total number of isolates; S - total number of groups formed; n_k - number of isolates belonging to group k.

Identification at the genus level was performed using a multiplex PCR method specific for *Leuconostoc* and *Lactobacillus* genera, based on genus-specific primers described in the literature (Dubernet *et al.*, 2002; Macián *et al.*, 2004). PCR reactions were performed in a final volume of 20 µl, containing 10 µl of NZYTaq 2× Green Master Mix (NZYtech, Portugal), 12.5 pmol each of primers LeucA, LeucS, LbLMA1-rev and R16-1 (StabVida, Portugal) (**Table 2**) and 1 µl of DNA. Amplification was performed using the following conditions: 5 min at 95°C; followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C; and a final extension step of 10 min at 72°C, followed by refrigeration at 4°C. PCR products were submitted to electrophoresis as described above. The presence of a 613 bp amplicon allowed identification of the isolates as *Leuconostoc* sp., whereas a 250 bp amplicon allowed identification as *Lactobacillus* sp. The technique was validated by testing relevant reference strains and the reproducibility was evaluated by performing 10% biological replicates.

Fisher's exact test was applied in order to determine if the distribution of *Lactobacillus* and *Leuconostoc* isolates in the fermentations was associated with either the type of cabbage used, or the inclusion of aromatic herbs and garlic at the start of fermentation. Statistical analysis was performed using R software.

Additionally, two more dendrograms were produced using the profiles of representative isolates from each genera. Groups of genomically similar isolates were defined, and the distribution of these groups throughout time was compared.

Table 2 - List of primers used in the present work.

Technique	Primer	Sequence (5'-3')	Amplicon length (bp)	Reference
PCR-Fingerprinting	BOXA1R	CTA CGG CAA GGC GAC GCT GAC G	]200-3000[	Gevers *et al.,* 2001
	ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	]200-3000[	Versalovic *et al.,* 1994
	ERIC2	AAG TAA GTG ACT GGG GTG AGC G		
	$(GTG)_5$	GTG GTG GTG GTG GTG	]200-3000[	Wouters *et al.,* 2013
	M13	GAG GGT GGC GGT TCT	]200-3000[	Cocolin *et al.,* 2004
	OPC-15	GAC GGA TCA G	]200-3000[	Vieira-Pinto *et al.*, 2008
	REP1R	III ICG UCG UCA TCI GGC	]200-3000[	Versalovic *et al.*, 1994
	REP2	ICG ICT TAT CIG GCC TAC		
Multiplex PCR	LbLMA1-rev	CTC AAA ACT AAA CAA AGT TTC	250	Dubernet *et al.,* 2002
	R16-1	CTT GTA CAC ACC GCC CGT CA		
	LeucA	CAC TTT GTC TCC GAA GAG	613	Macián *et al.,* 2004
	LeucS	AAG CAC TGT TGT ATG GGA		

2.3 - Safety evaluation

The selection of microorganisms for use as probiotic strains first requires the evaluation of their safety, including the presence of transferrable antimicrobial resistance or virulence factors.

The presence of hemolytic activity, a possible virulence factor, was evaluated by streaking in Columbia agar plates with 5% horse blood (Frilabo, Portugal). Plates were incubated for 24 h at 37°C. Isolates surrounded by a transparent halo were classified as β-hemolytic, and those surrounded by a green halo were classified as α-hemolytic; isolates presenting neither halo were classified as γ-hemolytic. *Aeromonas hydrophila* ATCC 7960 was used as a positive control (β-hemolytic), and *Enterococcus faecalis* CECT 795 was used as a negative control (γ-hemolytic).

Antimicrobial resistance profile of the isolates was determined using the disc diffusion method (CLSI, 2012). A list of the antimicrobial compounds used in this study is presented in **Table 3**. Briefly, isolates were grown in MRS agar overnight at 37°C; then, using a sterile swab, a suspension was prepared and adjusted to 0.5 McFarland standard (approximately 10^8 CFU/ml). Afterwards, the suspension was spread into MRS plates using a sterile swab, and antibiotic discs (Oxoid, UK) were placed on each one. Plates were incubated at 37°C and the diameter (mm) of inhibition halos was measured after 24 h of incubation. The mean (M) and standard deviation (SD) of the inhibition halo diameter (x) of all isolates were calculated for each antimicrobial compound, and isolates were classified as follows: $x \leq M-SD$ – resistant; $M-SD < x < M+SD$ – intermediate; $x \geq M+SD$ – susceptible.

L. rhamnosus GG was used as a probiotic control and its results were compared with the resistant/susceptible classification based in different methods used by other authors (Argyri *et al.*, 2013).

Fisher's exact test was applied in order to determine if the antimicrobial resistance of the isolates was associated with their genus, for each antimicrobial compound. Statistical analysis was performed using R software.

Table 3 - Concentration, class and mode of action of antimicrobial compounds studied in the present work.

Antimicrobial compound	Concentration (µg)	Antimicrobial compound class	Mode of action
Ampicillin	10	Penicillin	Cell wall synthesis inhibition
Vancomycin	30	Glycopeptide	
Chloramphenicol	30	Phenicol	Protein synthesis inhibition (50S inhibitors)
Clindamycin	2	Lincosamide	
Erythromycin	15	Macrolide	
Gentamicin	10	Aminoglycoside	Protein synthesis inhibition (30S inhibitors)
Kanamycin	30		
Streptomycin	10		
Tetracycline	30	Tetracycline	

2.4 - Characterization of probiotic potential

Three main characteristics were tested to investigate the probiotic potential of the representative isolates: 1) resistance to low pH and 2) resistance to bile, which are both adverse factors that may negatively impact probiotic viability and colonization of the human GI tract; and 3) antimicrobial activity against pathogenic microorganisms, which is desirable to inhibit enteropathogen growth and subsequent infection.

Resistance to low pH and bile were tested using a plate assay (Peres *et al.*, 2014). Briefly, isolates were grown in MRS broth (VWR, USA) at 37°C overnight, and then 5 µl of each bacterial culture were spotted in both MRS agar with pH 3.5, adjusted with HCl, and MRS agar supplemented with 0.5% bovine bile (Sigma, USA). Unmodified MRS plates were inoculated as control. Plates were incubated for 48 h at 37°C. Visible growth was recorded as positive for resistance to low pH or bile, and no growth as negative. Isolates positive to both characteristics were chosen for further analysis.

To confirm the resistance phenotype, selected isolates were further tested for resistance to low pH using a microplate assay. Isolates were grown in MRS broth overnight at 37°C. Bacterial cultures were then adjusted to approximately 10^{10} cells/ml using MRS broth, and 2 µl of these cultures were used to inoculate 198 µl of three media: MRS broth, pH 2.5; MRS broth, pH 3.0; and unmodified MRS broth. Each of these was performed in triplicate in a microplate, which was then incubated at 37°C for 24 h. After 3 h and 24 h of incubation, 5 µl from each suspension was spotted in MRS plates, which were incubated at 37°C for 24 h.

Isolates were also tested for antimicrobial activity against pathogenic bacteria (Bedin, 2014). *Listeria monocytogenes* CECT 935 was used as a pathogenic indicator. Isolates were streaked on MRS agar plates, and incubated at 37°C overnight. After incubation, a suspension of *Lis. monocytogenes* was prepared and adjusted to 5.0 McFarland standard (approximately 10^9 CFU/ml). Then, 100 µl of this suspension was used to inoculate 8 ml of molten Brain Heart Infusion (BHI) (VWR, USA) with 0.7% agar (VWR, USA), and the inoculated BHI medium was overlaid on top of the streaked MRS plates. After solidification, plates were incubated at 37°C overnight, and growth inhibition of the pathogenic indicator strain was recorded as positive for antimicrobial activity of the isolates.

An agar well diffusion assay (Argyri *et al.*, 2013; Botta *et al.*, 2014) was then performed in order to test if this antimicrobial activity was caused by extracellular compounds, secreted by the microorganisms. Briefly, isolates were grown in MRS broth at 37°C, overnight. Cell-free culture supernatants (CFCS) were obtained by centrifugation at 14000 rpm, 4°C, 15 min (Hermle® Z233 MK-2 centrifuge, Hermle, Germany) and then filtered using 0.22 µm filters (Merck Millipore, Germany), to eliminate any cells present. A suspension of *Lis. monocytogenes* was prepared and adjusted to 0.5 McFarland standard (approximately 10^8 CFU/ml). Then, 250 µl of the suspension was used to inoculate 25 ml of molten BHI with 1% agar and the inoculated media was plated in empty petri dishes. After solidification, 5 mm holes were performed in the agar, and 50 µl of CFCS were placed in each hole. Plates were incubated without inversion at room

temperature for 1 h, to allow diffusion of CFCS, and then inverted and incubated at 37°C, overnight. Inhibition halos surrounding the wells indicated that the antimicrobial activity against the pathogenic indicator was due to the presence of secreted compounds.

2.5 - Evaluation of pathogenic and probiotic characteristics using the *Caenorhabditis elegans* model

Two isolates were selected for the study using the *C. elegans* model. Analysis of their pathogenic and probiotic characteristics was based on the solid killing assay described by Kim and Mylonakis (Kim & Mylonakis, 2012). *C. elegans* strain N2 was used in this experiment. All plates containing worms were incubated at 20°C and 80% relative humidity. Observation and manipulation of nematodes was performed using a Nikon SMZ18 Stereo Microscope (Nikon Instruments Europe BV, Netherlands).

The experiment was performed by first feeding one of four microorganisms to the nematodes (conditioning): the selected LAB isolates L61 or L89; a probiotic control strain (*L. rhamnosus* GG); or a non-probiotic control strain, used in the normal maintenance of the worms (*E. coli* HT115). Afterwards, these worms were fed one of two strains (testing): either a strain with pathogenic effects (*Serratia* sp.); or a non-pathogenic strain (*E. coli* OP50), which served as a negative control for the test.

At the start of the assay, synchronization of *C. elegans* populations was done in order to guarantee that every worm submitted to this protocol was at the same stage of their life cycle. This was performed by picking 20 adult worms to each conditioning plate, which were allowed to lay eggs for approximately five hours, and were then removed or killed.

Conditioning to a strain was performed by growing the synchronized nematode population since hatching until adulthood (approximately three days) using that strain as the only food source. Afterwards, two adult worms were picked from each of the conditioning plates to testing plates of either *E. coli* OP50 or *Serratia* sp., five plates being performed in this way for each conditioning/test combination. Worms were observed regularly and picked to new plates with the same microorganism daily until the fourth day of adulthood, and every two or three days afterwards until all worms were dead. The number of descendants for each plate was counted, and dead worms and their respective time of death were observed. A representation of the experimental design is shown in **Figure 4.**

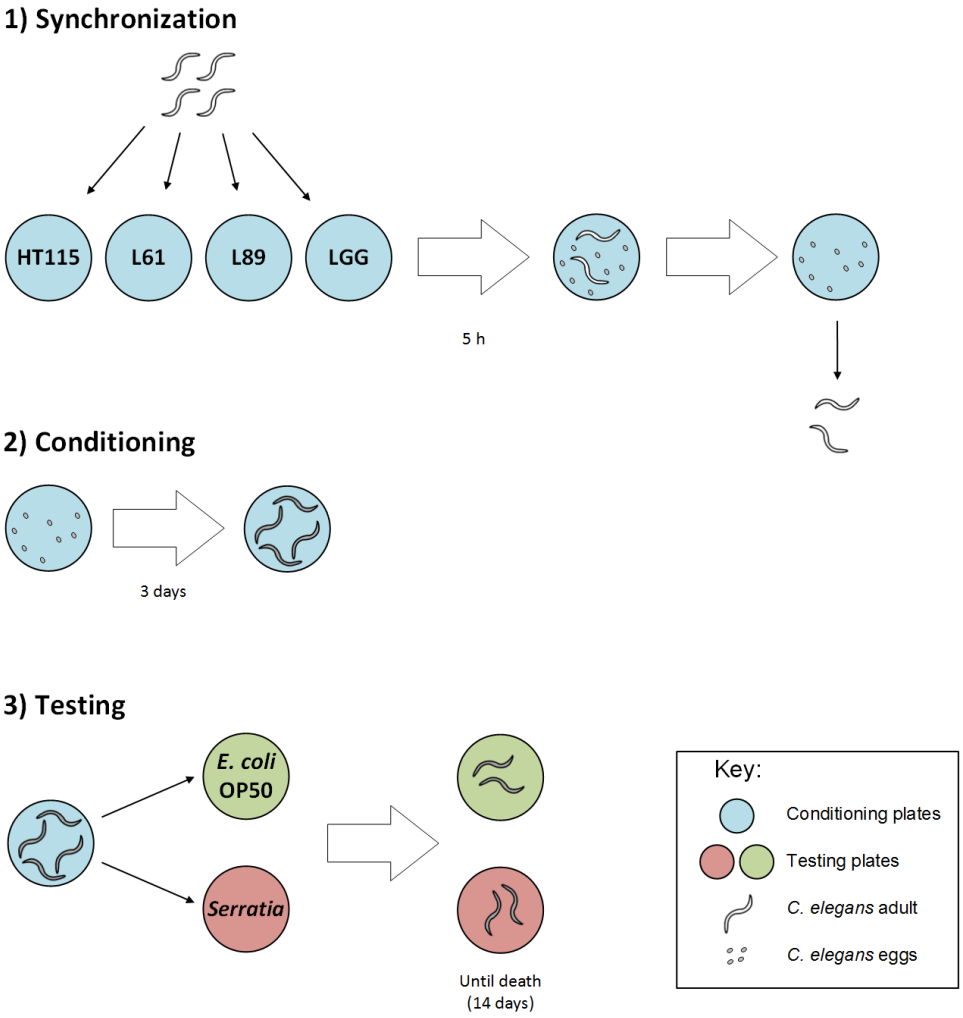

Figure 4 - Experimental design used for the analysis of pathogenic and probiotic characteristics of the isolates under study. Synchronization of *C. elegans* populations was performed (**1**) and worms were first fed with one of four strains: *E. coli* HT155, L61, L89 or *L. rhamnosus* GG (LGG) (**2** - conditioning). These were then transferred to new plates and fed with either *Serratia* sp. or *E. coli* OP50 (**3** - testing).

For the preparation of conditioning and testing plates, bacteria were grown in either Luria Broth (LB) with agitation, for *E. coli* and *Serratia* sp., or MRS broth without agitation, for LAB, at 37°C overnight. Conditioning plates were prepared by spreading 100 µl of either *E. coli* HT115, isolates L61 or L89 or *L. rhamnosus* GG on 90 mm sized Nematode Growth Medium (NGM) plates. Testing plates were prepared by spotting 5 µl of either *E. coli* OP50 or *Serratia* sp. in the center of 60 mm sized NGM plates with 100 µg/ml ampicillin. Ampicillin was added to the testing plates to avoid contamination with the strains used in conditioning, since these were sensitive to ampicillin and the testing strains were ampicillin resistant. Both conditioning and testing plates were incubated at 37°C overnight and then stored at 4°C until use.

Quantification of microorganisms present in the nematode gut was also performed, according to Kim and Mylonakis (Kim & Mylonakis, 2012). Briefly, worms from the conditioning plates were picked to bacterial lawns of *E. coli* OP50 or *Serratia* sp. on NGM (Europa Bioproducts,

UK) 90 mm plates with 100 µg/ml ampicillin, and transferred to new plates every two days. At days 0, 2 and 4, at least three individual nematodes were taken from each plate and washed on 12- or 24-well plates twice with M9 medium + 1% Triton-X, and once with the same solution containing 25 µg/ml of gentamicin, in order to eliminate surface bacteria. Each individual nematode was then placed in a sterile tube and crushed using a pestle. Afterwards, 500 µl of M9 medium was added to the tube, and two decimal dilutions were performed, in triplicate. The 10^{0}, 10^{-1} and 10^{-2} dilutions were then inoculated by spotting 5 µl on both MRS and either LB plates (day 0) or LB plates with 100 µg/ml ampicillin (days 2 and 4). LB and LB + ampicillin plates were incubated at 37°C for 12 to 24 h, and MRS plates were incubated at 37°C for 24 to 48 h.

Statistical analysis of total fertility and fertility per day was performed by two-way Analysis of Variance (ANOVA) test and Fisher's exact test, respectively (n=2, 5 replicates). For survival analysis, all individual nematodes from each plate were considered as part of only one replicate (n=10), and data was right censored. Survival analysis was performed by modelling the data using the Kaplan-Meyer estimator to obtain survivorship curves, and the Cox proportional hazards regression model was used to calculate the hazard ratios between nematodes in different conditions and tests. All statistical analysis was performed using R software.

3 - Results and discussion

In order to isolate microorganisms with putative probiotic potential, the following methodology was performed. First, sauerkraut fermentations were performed/studied, and LAB were isolated from those fermentations. Then, isolates were characterized phenotypically and genotypically and their safety and probiotic potential was assessed *in vitro*. Finally, selected isolates were tested using the *C. elegans* model, in order to evaluate their pathogenic and probiotic potential *in vivo*.

3.1 - Sauerkraut fermentations

Initially, 18 sauerkraut fermentations (six different recipes, each executed in triplicate) were performed. pH, titratable acidity and viable LAB quantity were assessed and LAB were isolated from all 18 fermentations during a time span of 30 days (data not shown). Although all fermentations showed similar profiles regarding these three parameters, it was necessary to reduce their number to proceed with further testing, and so four fermentations with adequate profiles were chosen. The selected fermentations consisted on two pointed-head cabbage and two portuguese cabbage fermentations, with and without aromatic herbs added to their recipe, and their pH, acidity, and LAB quantity profiles are shown in **Figures 5, 6 and 7**, respectively.

The pH value of the fermentation juices (**Figure 5**) at the start of the fermentations was slightly acidic (between 5.5 and 6). For the portuguese cabbage fermentations, a sharp decrease of the pH value was observed until day 7, which then increased slightly until the end of the fermentation. A similar result was observed for the pointed-head cabbage fermentations. Although in the latter case the pH values of the earlier time-points were not measured, a low value of pH was reached by day 16, which either stabilized or increased slightly until the end of fermentation.

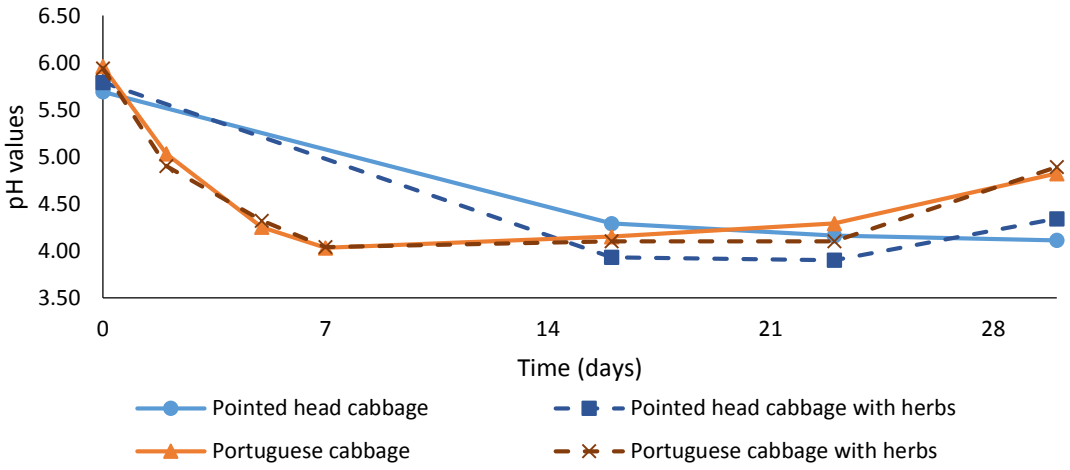

Figure 5 – Variation of pH values along fermentation time, for the four chosen fermentations.

Regarding the acidity of fermentation juices (**Figure 6**), for the portuguese cabbage fermentations there was an increase in the percentage of acid measured until day 7, varying during the rest of the fermentation process; no value higher than the one recorded in day 7 was observed. For the pointed-head cabbage fermentations, the highest observed value occurred at day 23. However, it is possible that a similar peak may have occurred at earlier time-points, since no acidity quantification was performed between days 0 and 16 for these two fermentations.

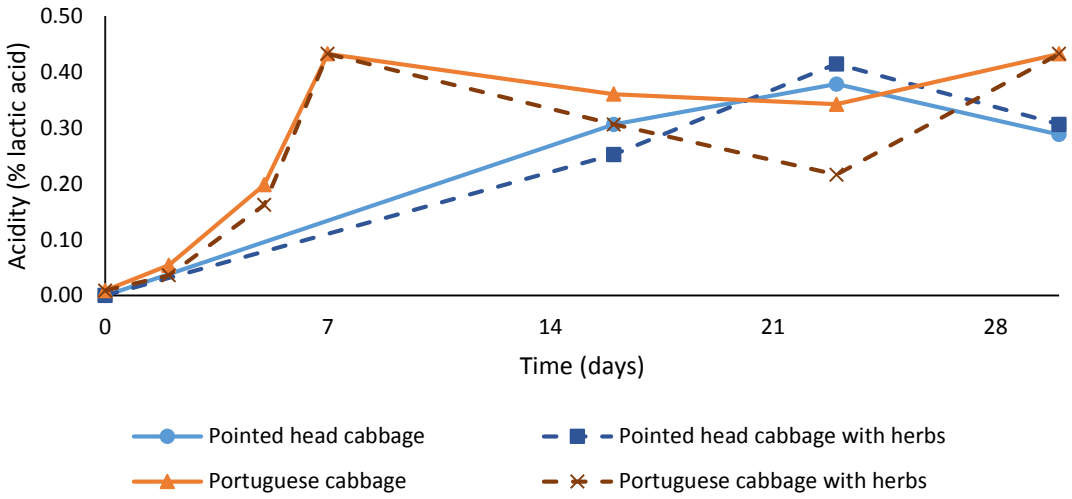

Figure 6 - Variation of acid content along fermentation time, for the four chosen fermentations.

Finally, the fermentation substrates had a low number of culturable LAB cells, below the detection limit. A great increase in the quantity of LAB was observed during the first two days, which then stabilized and decreased slightly from day 7 onwards (**Figure 7**).

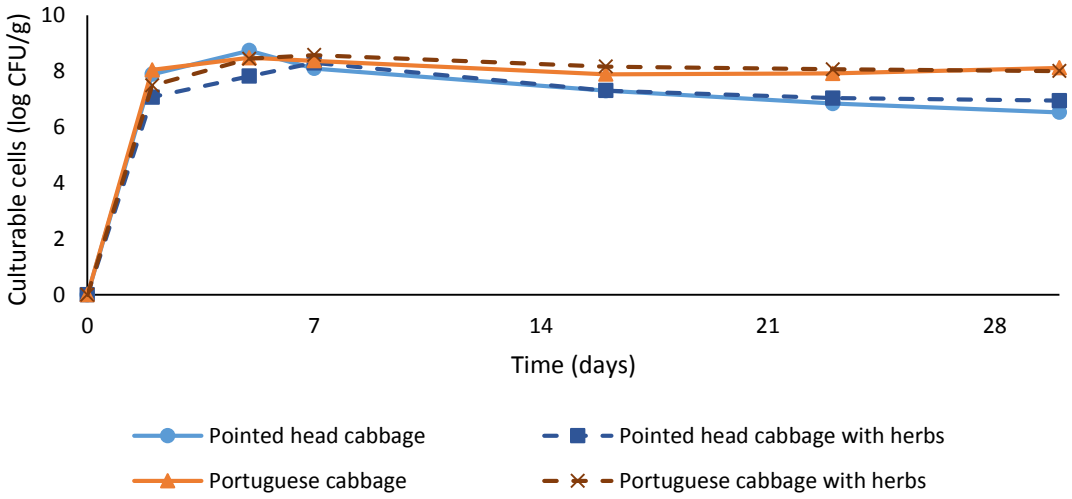

Figure 7 - Variation in culturable lactic acid bacteria quantification along fermentation time, for the four chosen fermentations.

Most of the chemical and microbiological changes in fermentations occurred in the first days, particularly until day 7. All four fermentations showed comparable patterns in the measured parameters, with fermentations of the same type of cabbage having more similar results, despite differing on the presence or absence of aromatic herbs.

Profiles based on the measured parameters are generally in agreement with those previously described for sauerkraut fermentations, though some differences should be noted. The fermentations from the present work reached a lower percentage of acidity (0.3 to 0.4%) and a higher pH value (4.1 to 4.9) than what is traditionally obtained at similar temperatures (1.6 to 2.3% and 3.5 or less, respectively) (Harris, 1998; Lu *et al.*, 2003).

This discrepancy can be explained by differences in sauerkraut fermentation conditions, particularly the substrates used. Different varieties of cabbage, or even different cultivars of the same variety, may have a different composition of nutrients, such as soluble sugars (Rosa *et al.*, 2001). In fact, it has been observed that white cabbage has a higher quantity of sugar than some portuguese cabbage cultivars (Rosa *et al.*, 2001). The level of this nutrient present on the substrate may be particularly important, since the presence of a low quantity of sugar will lead to less acid production during the fermentation process.

The differences may also have been caused by the addition of a NaCl solution to the substrate prior to the fermentation. This step is not usually performed in sauerkraut fermentations and may have diluted the soluble cabbage nutrients, resulting in lower sugar and, consequently, lower acid concentrations. The addition of this solution was performed due to limitations on the quantity of substrate available, and in order to guarantee an anaerobic environment in the fermentation vessel.

Despite the difference in the maximum values of acidity and pH, the evolution of the three parameters through the timespan followed the patterns described in the literature, quickly changing in the first days and then stabilizing (Holzapfel *et al.*, 2008). This suggests that the sauerkraut fermentations were probably performed correctly, and were appropriate for the isolation of LAB.

3.2 - Isolation, characterization and identification of lactic acid bacteria

Lactic acid bacteria were isolated from the 18 sauerkraut fermentations at the start of, and after 2, 5, 7, 16, 23 and 30 days of fermentation, with a collection of 301 isolates being obtained. In order to identify and characterize microorganisms with putative probiotic potential, an adequately sized subgroup of isolates was required, and therefore those from the previously chosen four fermentations were selected for a more detailed analysis. This group included 114 LAB isolated from fermentations with different recipes, which increased its microbial diversity. Information relative to the origin of these isolates is included in **Table 4**.

Table 4 – Number of microorganisms isolated from each time-point for the four sauerkraut fermentations.

Fermentation	Time (days)							Total
	0	2	5	7	16	23	30	
Portuguese cabbage	5	5	5	5	5	5	5	35
Portuguese cabbage with herbs	3	4	2	5	5	5	5	29
Pointed-head cabbage	0	0	5	5	5	5	5	25
Pointed-head cabbage with herbs	0	0	5	5	5	5	5	25
								114

Phenotypic characterization, including Gram staining, oxidase and catalase tests, was first performed in order to exclude microorganisms that did not present a LAB-like phenotype. Results showed that 95 of the isolates were Gram-positive, oxidase-negative and catalase-negative (or weakly positive, in one case), phenotypic characteristics associated with LAB.

Subsequently, PCR-fingerprinting technique was used to select representative isolates and exclude clones. For this purpose, six reaction mixtures were tested, based on eight previously described primers (**Table 2**, page 19). The reaction mixtures based on primers M13 and OPC-15 generated profiles with a higher number of bands and allowed a better discrimination of different reference strains, and so were applied for all isolates.

Thus, PCR-fingerprinting was performed for the 95 isolates using primers M13 and OPC-15 and the banding profiles obtained were analyzed using BioNumerics software (version 6.6.5), and compared by dendrogram analysis. To analyze the technique reproducibility, a first dendrogram was constructed using the profiles of the replicates and the corresponding isolates, and the average reproducibility level obtained was 83.3% (data not shown).

A second dendrogram was constructed with the fingerprinting profiles of all isolates (**Figure 8**). Microorganisms with similarity above the reproducibility level were considered genomically similar until proven otherwise. Representative isolates corresponding to each different time-point/fermentation were chosen among those genomically similar, with a total of 63 representatives being selected.

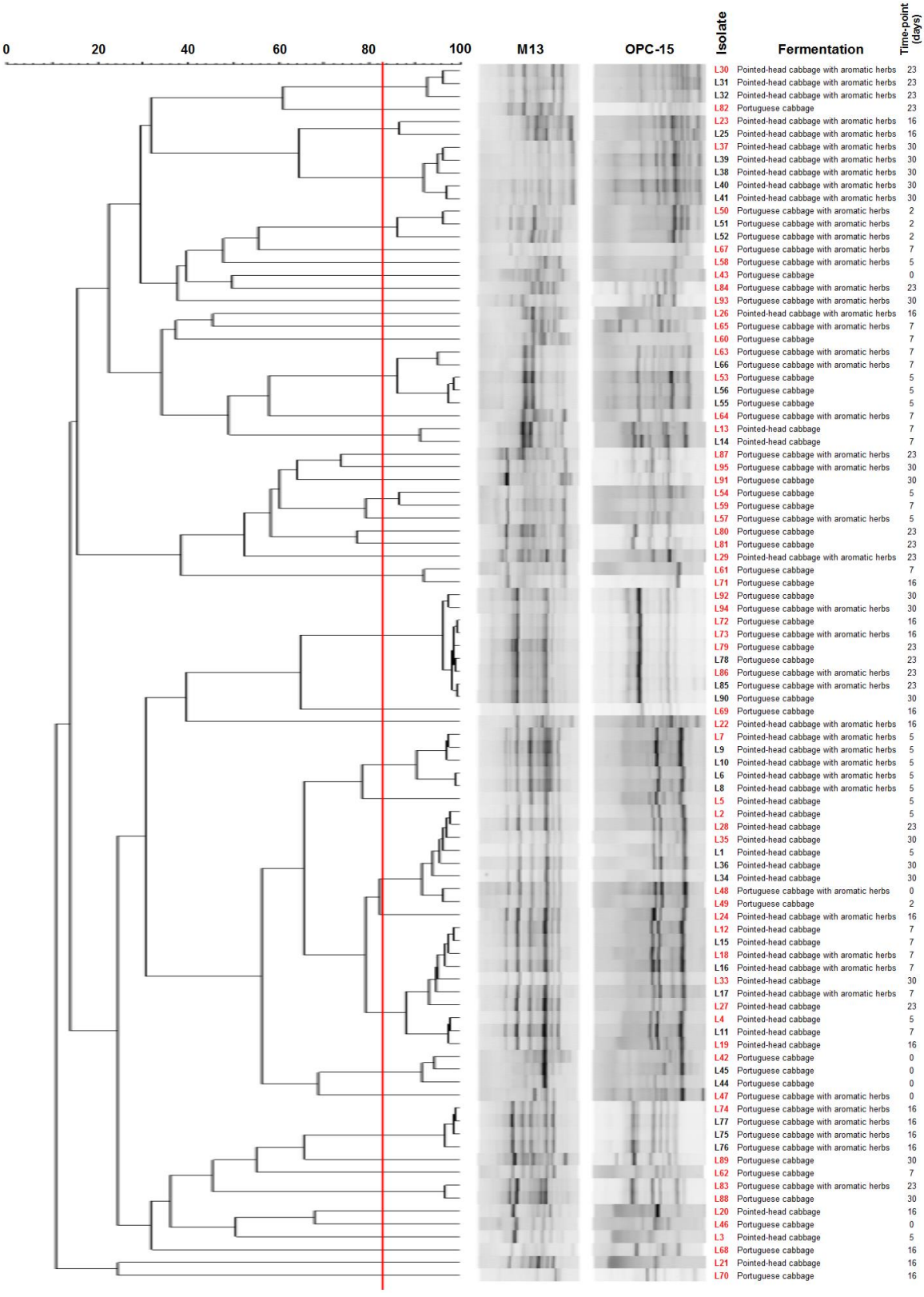

Figure 8 – Dendrogram constructed using the M13 and OPC-15 PCR-fingerprinting profiles of the 95 isolates from the four sauerkraut fermentations. The red vertical line represents the reproducibility level, which was used as a cut-off value for the definition of representative isolates. Isolates written in red were chosen as representatives for further studies.

Furthermore, in order to calculate the diversity of LAB present throughout the fermentation process, dendrograms based in the profiles of all isolates from each fermentation were also obtained (data not shown). Diversity was assessed by the Simpson's diversity index (D'), which indicates the probability of two randomly selected isolates belonging to different groups, and is a measure that takes into account both the richness and the evenness of the groups analyzed (Hunter & Gaston, 1988). A higher D' value indicates a higher diversity, whereas a lower D' value indicates a lower diversity.

Both portuguese cabbage fermentations had similar diversity (D'=0.96), while pointed-head cabbage fermentations, with or without aromatic herbs, had lower diversity (D'=0.87 and 0.77, respectively). Diversity seemed to differ between each fermentation recipe, with the variety of cabbage used having a greater effect than the inclusion of aromatic herbs. This result may be due to the different characteristics of each substrate, such as nutrient quantity/composition (Rosa *et al.*, 2001), which may affect the fermentation, its microbial community and therefore its total LAB diversity.

The 63 representative isolates were then identified to the genus level by multiplex PCR, using two genus-specific primer pairs (**Table 2**, page 19). First, a protocol optimization was performed with reference strains belonging to the target genera, as well as strains belonging to two LAB genera closely related to the targets, *Pediococcus* and *Lactococcus,* in order to confirm the specificity of the reaction. The results showed that the reaction successfully amplified the appropriate fragment for the genus of each reference strain, with *Pediococcus* and *Lactococcus* species showing no amplification, as expected (data not shown).

After optimization, multiplex PCR was performed for the representative isolates. From the 63 isolates, 21 were identified as *Leuconostoc* spp. (33%, with a 250 bp amplicon) and 33 as *Lactobacillus* spp. (52%, with a 613 bp amplicon), while nine isolates remained unidentified (14%, with no amplicons) by this method. A reproducibility of 100% was achieved for this technique. The distribution of the two genera was not uniform among sauerkraut fermentations (**Figure 9**). While the majority of isolates in pointed-head cabbage fermentations were identified as *Leuconostoc* (n=16 out of 22), in portuguese cabbage fermentations most isolates belonged to the *Lactobacillus* genus (n=31 out of 41).

In order to test the significance of this difference, the isolates from the two pointed-head cabbage fermentations were compared to those from the two portuguese cabbage fermentations, using Fisher's exact test. The two groups were shown to be significantly different from each other ($P<0.001$). Furthermore, isolates from the fermentations performed with aromatic herbs were also compared to those performed without their addition using the same test, and the results were not significantly different ($P<0.05$). These results indicate that the distribution of *Lactobacillus* and *Leuconostoc* genera in sauerkraut fermentations is dependent on the type of cabbage used as substrate, but not on the addition of aromatic herbs to the recipe.

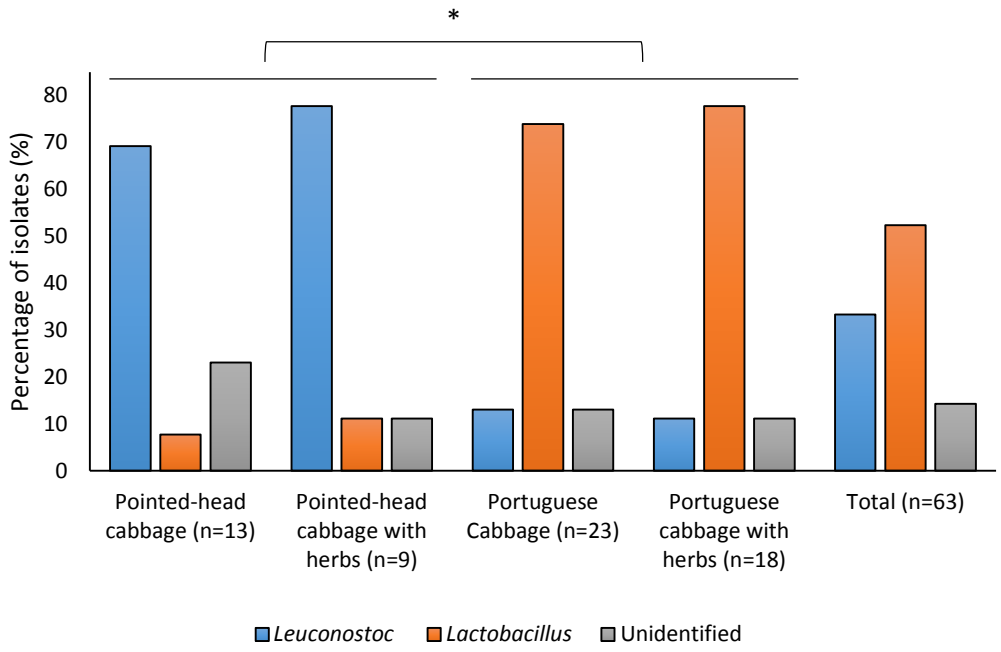

Figure 9 – Incidence of identified *Leuconostoc* and *Lactobacillus* isolates among the representatives from the four different sauerkraut fermentations. * - $P<0.001$

After identification, a new dendrogram was created for each genus (**Figure 10**). Groups of genomically similar bacteria were defined using the reproducibility level (83.3%) as a cut-off value. The analysis of groups of strains belonging to the same genus allowed the definition of more accurate groups. Therefore, four isolates previously considered as representatives were grouped with others of the same fermentation/time-point and were excluded from additional studies.

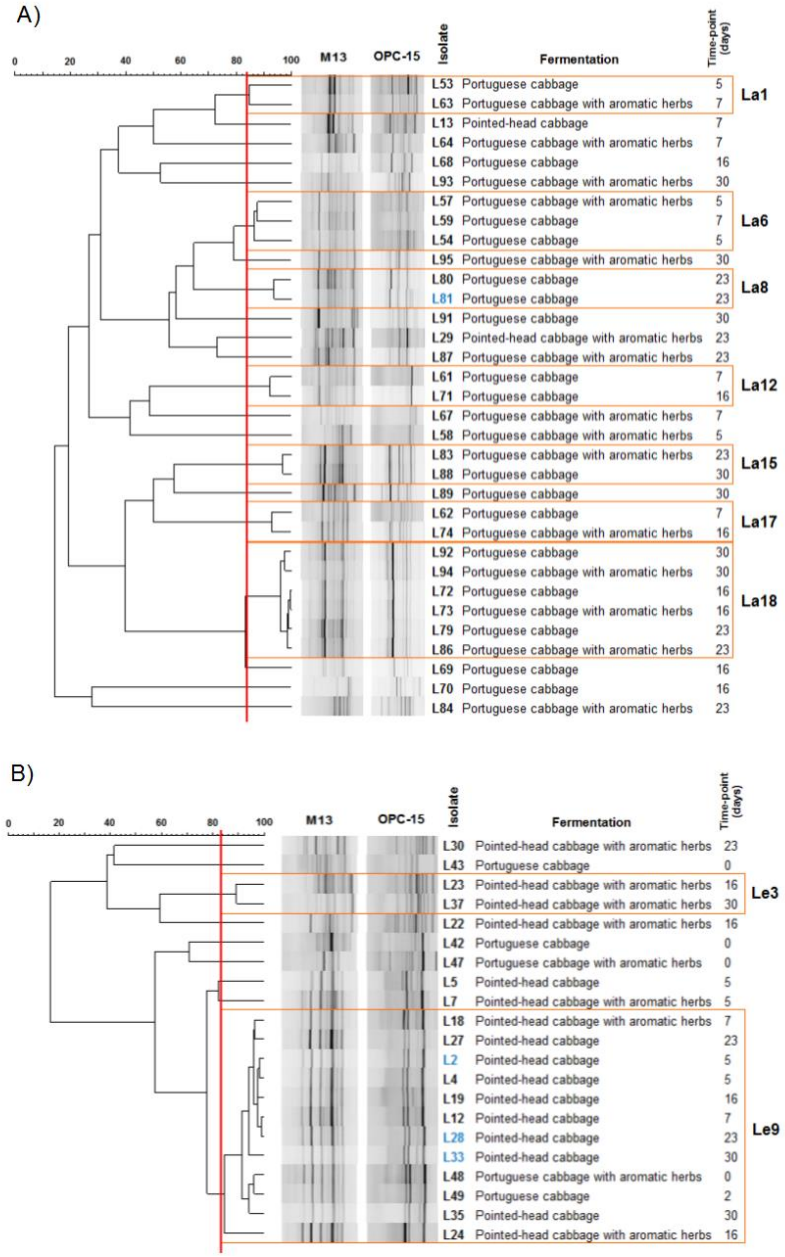

Figure 10 – Dendrograms constructed using the PCR-fingerprinting profiles of the *Lactobacillus* (**A**) or *Leuconostoc* (**B**) isolates. The red vertical line represents the reproducibility level, which was used as a cut-off value for the definition of genomically similar groups. Groups containing more than one isolate are represented. Isolates written in blue were considered genomically similar to others of the same fermentation/time-point until proven otherwise, and were removed from subsequent testing.

Analysis of groups shared between the fermentations (**Table 5**) allowed several observations. First, microorganisms belonging to one specific cluster of *Leuconostoc*, Le9, were present at early time-points in every fermentation. Furthermore, in the pointed-head cabbage fermentation without herbs, microorganisms from this cluster persisted in every time-point and dominated the fermentation in comparison with other groups.

On the contrary, the other three fermentations showed a more diverse distribution of microbial clusters. Several groups were present in both portuguese cabbage fermentations, with

one group of *Lactobacillus* in particular, La18, being present in all time-points from day 16 onwards. This may indicate the importance of the microorganisms from this group to the fermentation process.

Table 5 - Presence of clusters of *Leuconostoc* (Le) and *Lactobacillus* (La) isolates over the course of the four chosen fermentations. Groups in bold were present in more than a single time-point, in the same or in distinct fermentations.

Time (days)	Pointed-head cabbage	Pointed-head cabbage with herbs	Portuguese cabbage	Portuguese cabbage with herbs
0	-	-	Le2, Le5	Le6, **Le9**
2	-	-	**Le9**	-
5	Le7, **Le9**	Le8	**La1, La6**	**La6**, La14
7	**Le9**, La10	**Le9**	**La6, La12, La17**	**La1**, La3, La13
16	**Le9**	**Le3**, Le4, **Le9**	La4, **La12**, **La18**, La19, La20	**La17, La18**
23	**Le9**	Le1, La12	La8, **La18**	La11, **La15**, **La18**, La21
30	**Le9**	**Le3**	La9, **La15**, La16, **La18**	La5, La7, **La18**

Results observed for the portuguese cabbage fermentations are in accordance with the work of Plengvidhya and coworkers (2007), which characterized the distribution of species across 14 days for four sauerkraut fermentations, by 16S RNA sequencing of isolates. They found that for three fermentations, the majority of microorganisms isolated until the third day of fermentation belonged to *Weissella* and *Leuconostoc* genera, and for the seventh and fourteenth day the majority of microorganisms isolated were from the *Lactobacillus* genus, particularly from the *L. plantarum* species. In the present work, the same distribution was observed in the portuguese cabbage fermentations, with *Leuconostoc* being isolated at the start and at the second day of fermentation, and *Lactobacillus* from the fifth day onwards.

Results for the pointed-head cabbage fermentations showed a different LAB distribution, with a predominance of *Leuconostoc* spp. at every time-point, which is not usually reported for sauerkraut fermentations. However, Plengvidhya and coworkers (2007) also observed a different pattern of microbial groups in one of the fermentations analyzed, with both hetero- and homofermentative species (normally isolated at the early and late stages of fermentation, respectively) being present at every time-point, which may indicate that different patterns of microorganisms can occur in sauerkraut fermentations. Variations found between the various types of fermentations are probably due to differences in chemical, biochemical and/or microbiological characteristics between the varieties of cabbage used as substrate. This is supported by the previously stated fact that different varieties or cultivars may have differing nutrient compositions (Rosa *et al.*, 2001), which may affect the microbial succession.

3.3 - Safety evaluation and characterization of probiotic potential

After genus identification, representative isolates were first tested for the presence of antimicrobial resistance, hemolytic activity and resistance to low pH and bile. Then, a group of fewer isolates was selected based on those characteristics, and tested for resistance to a lower pH value and for antimicrobial activity against *Listeria monocytogenes*.

It is important to assess the safety of microorganisms to be used as probiotics, in order to avoid any potential infections and unwanted side-effects, particularly in immunosuppressed individuals, and also to prevent the possibility that these microorganisms could act as reservoirs for antimicrobial resistance gene dissemination.

One possible virulence factor that can be present in pathogenic microorganisms and is usually evaluated in probiotic candidates is the ability to lyse erythrocytes – hemolysis. From the tested isolates (n=59), only one was considered β-hemolytic, with 18 presenting α-hemolysis and 40 having a γ-hemolytic phenotype.

These results are in accordance with previous studies that isolated probiotic microorganisms from different vegetable sources with no β-hemolytic activity and few presenting α-hemolytic activity (Argyri *et al.*, 2013; Botta *et al.*, 2014). α-hemolysis has been considered safe in non-enterococcal LAB by some authors (Argyri *et al.*, 2013; Lee *et al.*, 2011). This fact, coupled with the low incidence of β-hemolysis, indicated that the majority of isolates in the present work could be considered safe and may be used as probiotics.

To study the antimicrobial resistance of microorganisms, it is first important to clearly establish the breakpoint values used for their classification as resistant or non-resistant. The Clinical and Laboratory Standards Institute and related institutions have not defined breakpoints for the study of antimicrobial resistance in *Lactobacillus* and *Leuconostoc* species by disc diffusion (CLSI, 2012), and there is not a clear consensus on the values to be used in these cases. Some works use the breakpoints followed by Charteris and coworkers (1998), but these were not accessible for use in the present work. Therefore, breakpoints were defined for each antimicrobial based on the resistance level of all isolates in study. Isolates presenting an inhibition halo diameter equal or below the mean minus standard deviation of all isolates were considered resistant, while those with a diameter above this value were considered sensitive or intermediate (non-resistant).

The results for antimicrobial resistance are shown in **Figure 11**. A low percentage of LAB isolates were classified as resistant to ampicillin (12%), chloramphenicol (15%) and clindamycin (19%), but almost all isolates were resistant to vancomycin (90%). For the other antimicrobial compounds tested, the results were genus-dependent ($P<0.05$). For erythromycin and tetracycline, 22 and 34% of the *Lactobacillus* isolates were classified as resistant, respectively, while no *Leuconostoc* isolate was resistant to these antimicrobials. A high number of *Lactobacillus* spp. were resistant to gentamicin (66%), kanamycin (100%) and streptomycin (94%), while *Leuconostoc* spp. presented absent, low (22%) and medium (39%) levels of resistance,

respectively. *Lactobacillus rhamnosus* GG, a widely studied probiotic strain, was used as a control, and showed resistance to vancomycin and kanamycin.

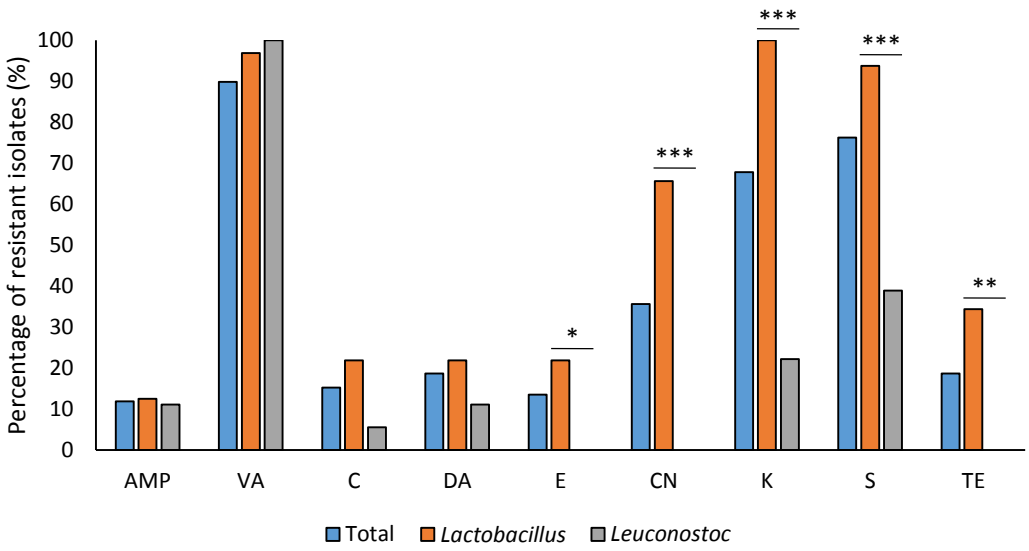

Figure 11 - Percentage of isolates resistant to the studied antimicrobial compounds. AMP- Ampicillin; VA- Vancomycin; C- Chloramphenicol; DA- Clindamycin; E- Erythromycin; CN- Gentamicin; K- Kanamycin; S- Streptomycin; TE- Tetracycline. * - P<0.05; ** - P<0.005; ***-P<0.0005

The high incidence of vancomycin resistance in *Lactobacillus* and *Leuconostoc* genera, observed in the present work, is a well-established phenomenon. This resistance is intrinsic, chromosomally encoded and caused by the presence of a different dipeptide (D-Ala-D-lactate instead of D-Ala-D-Ala) in the cell wall, which is the target for the action of this drug (Ammor *et al.*, 2007). Due to the intrinsic nature of this resistance, its risk of transmissibility is negligible and its presence should not be used as a criteria for the exclusion of isolates as probiotic candidates.

A high percentage of resistance to gentamicin, kanamycin and streptomycin was also detected in *Lactobacillus* spp. These antimicrobials are aminoglycosides, to which lactobacilli have been described as having a high natural resistance (Bernardeau *et al.*, 2008). Resistance to this class of antimicrobial compounds is common, can be considered intrinsic, and is believed to occur due to the absence of cytochrome-mediated electron transport, which mediates drug uptake by the microorganisms (Ammor *et al.*, 2007; Hummel *et al.*, 2007). The *Leuconostoc* genus is usually reported to be resistant to aminoglycosides (Ammor *et al.*, 2007). However, in the present work no resistance to gentamicin was found in isolates of this genus, results which are corroborated by other authors (Ogier *et al.*, 2008). Similarly to vancomycin resistance, the risk of transmissibility of aminoglycoside resistance is low or absent, so its presence can be considered safe and not an excluding characteristic for probiotic candidate selection.

For the other antimicrobials studied, *Lactobacillus* isolates showed a low level of resistance. Resistance to these antimicrobials is not widespread in this genus, although it has been linked to transmissibility to other microorganisms: resistance to ampicillin can be caused by point mutations, but may also be caused by the presence of the *blaZ* gene, which has been found

in transposons in *Lactobacillus*; the *cat* gene, which is present in plasmids, and the *lnu(A)* gene, which may be found in plasmids in staphylococci, confer chloramphenicol and lincosamide resistance respectively, and have also been reported as present in *Lactobacillus* strains; finally, several different genes from the *tet* and *erm* gene families have been found in plasmids and transposons in this genus, with *tet(M)* and *erm(B)* being the most common of these (Ammor *et al.*, 2007; Devirgiliis *et al.*, 2013; Fraqueza, 2015; Kastner *et al.*, 2006). Therefore, isolates presenting resistance to these antimicrobials may possess these genes, and act as reservoirs for their dissemination. Taking this into account, 42% of the isolates (n=25 of 59) were resistant to at least one of these antimicrobial compounds, and were later not selected for further testing.

Incidence of antimicrobial resistance in the studied isolates is supported by similar findings in LAB isolated from vegetable fermentations, despite the use of different techniques. Botta and coworkers (2014) reported resistance to gentamicin and kanamycin, and susceptibility to ampicillin, chloramphenicol, clindamycin, erythromycin and tetracycline in LAB isolates. Argyri and coworkers (2013) showed comparable resistance profiles, but variable resistance to ampicillin, gentamycin and streptomycin, and a low level of resistance to chloramphenicol and tetracycline.

These studies characterized isolates from fermented olives and used a micro-dilution broth technique for testing antimicrobial resistance, but despite the differences, the results were similar to the present work. Furthermore, the resistance profile of *L. rhamnosus* GG was also comparable to that obtained by Argyri and coworkers (2013), only differing in ampicillin resistance, which indicates that profiles obtained by different methodologies may be comparable. On the other hand, Beganović and coworkers (2014) performed the same methodology that was used in the present study, and reported resistance to tetracycline and no resistance to gentamicin and streptomycin in LAB isolated from fermented sauerkraut. These differences may be explained by the low number of isolates used in that work (five).

It is important to note that the breakpoints used in this work may be influencing the number of isolates classified as resistant. Due to the lack of established breakpoints in the literature and the definition of specific breakpoints for the present work based on a restricted collection of isolates, these do not take into account the real level of antimicrobial resistance of a microbial group or genus. Furthermore, if the majority of isolates are very sensitive to an antimicrobial compound, the breakpoints may be overestimated, leading to a misrepresentation of the level of resistance. In fact, this may be the case for some of the antimicrobials tested, such as ampicillin and chloramphenicol.

After evaluating the safety profile of the probiotic candidates, isolates were tested for resistance to low pH and bile, since they have to resist these harsh conditions in order to colonize the human GI tract and exert their health benefits. For this purpose, an agar based screening protocol was performed, with results showing that few isolates were resistant to low pH conditions (20%, n=12 out of 59), none of these belonging to the *Leuconostoc* genus. Furthermore, a high number of isolates were resistant to bile (88%, n=52 out of 59). *L. rhamnosus* GG was used as a probiotic control, and was resistant to both 0.5% bile and to a pH of 3.5.

Results from other authors showed variable results regarding bile resistance of LAB isolated from vegetable fermentations. Most studies, but not all, showed high incidence of resistance to bile or bile salts (Argyri *et al*., 2013; Chang *et al*., 2010; Ji *et al*., 2015; Yu *et al*., 2013), corroborating the results found in the present work.

Once this initial screening was performed, isolates were chosen based on the absence of hemolytic activity, antimicrobial resistance and resistance to low pH and bile (**Figure 12**). Six isolates were selected: L54, L59, L61, L71, L80 and L89, all belonging to the *Lactobacillus* genus. These were then tested for antimicrobial activity against *Listeria monocytogenes* and resistance to a lower pH than previously tested.

Listeria monocytogenes is a Gram-positive foodborne pathogen that can cause infection in pregnant women and their fetus and various conditions in other individuals, representing a cause for large foodborne outbreaks and related mortality (Gahan & Hill, 2014). Recently there was an outbreak of *Lis. monocytogenes* in Portugal, which demonstrates its impact in the public health (Magalhães *et al.*, 2015).

All six isolates were shown to have antimicrobial activity against *Lis. monocytogenes* in a spot-on-lawn assay, but when the inhibitory activity of culture supernatants was tested in an agar well diffusion assay, inhibition was non-existent or very weak.

These results show that the tested isolates inhibited *Lis. monocytogenes*, but the nature of this effect is not completely clear. It is evident that this antibacterial effect was not caused by any soluble compound present after growth of the isolates in liquid media, such as organic acids or bacteriocins, since these would lead to growth inhibition in the well diffusion assay. However, antimicrobial activity was observed on the spot-on-lawn test, with the presence of inhibition halos surrounding the isolates grown in solid media. A possible explanation is that the production of inhibitory compounds was induced by the presence of the pathogen, since inhibition was only shown when the isolates and the pathogen where in direct contact. In fact, in some cases co-culture of lactic acid bacteria with target cells can be a requirement for induction of bacteriocin production (Cotter *et al.*, 2005).

Antimicrobial activity of LAB, particularly bacteriocin production, is a strain specific characteristic. Studies on this feature have shown different results. Argyri and coworkers (2013) found that pH neutralized cell-free culture supernatants of 71 LAB isolates from fermented olives showed no antibacterial activity against *Lis. monocytogenes* and other microorganisms in an agar well diffusion assay, which indicates that these strains are not bacteriocin producers. Botta and coworkers (2014) reported that 22 out of 238 LAB showed antibacterial activity through a similar assay, but further testing attributed this activity to organic acid production, and not to bacteriocin-like compounds.

The results described in the present work, coupled with findings by other authors, may indicate that bacteriocin production against *Lis. monocytogenes* is not common in LAB isolated from fermented vegetables. In conclusion, there is strong evidence of the antibacterial effect of

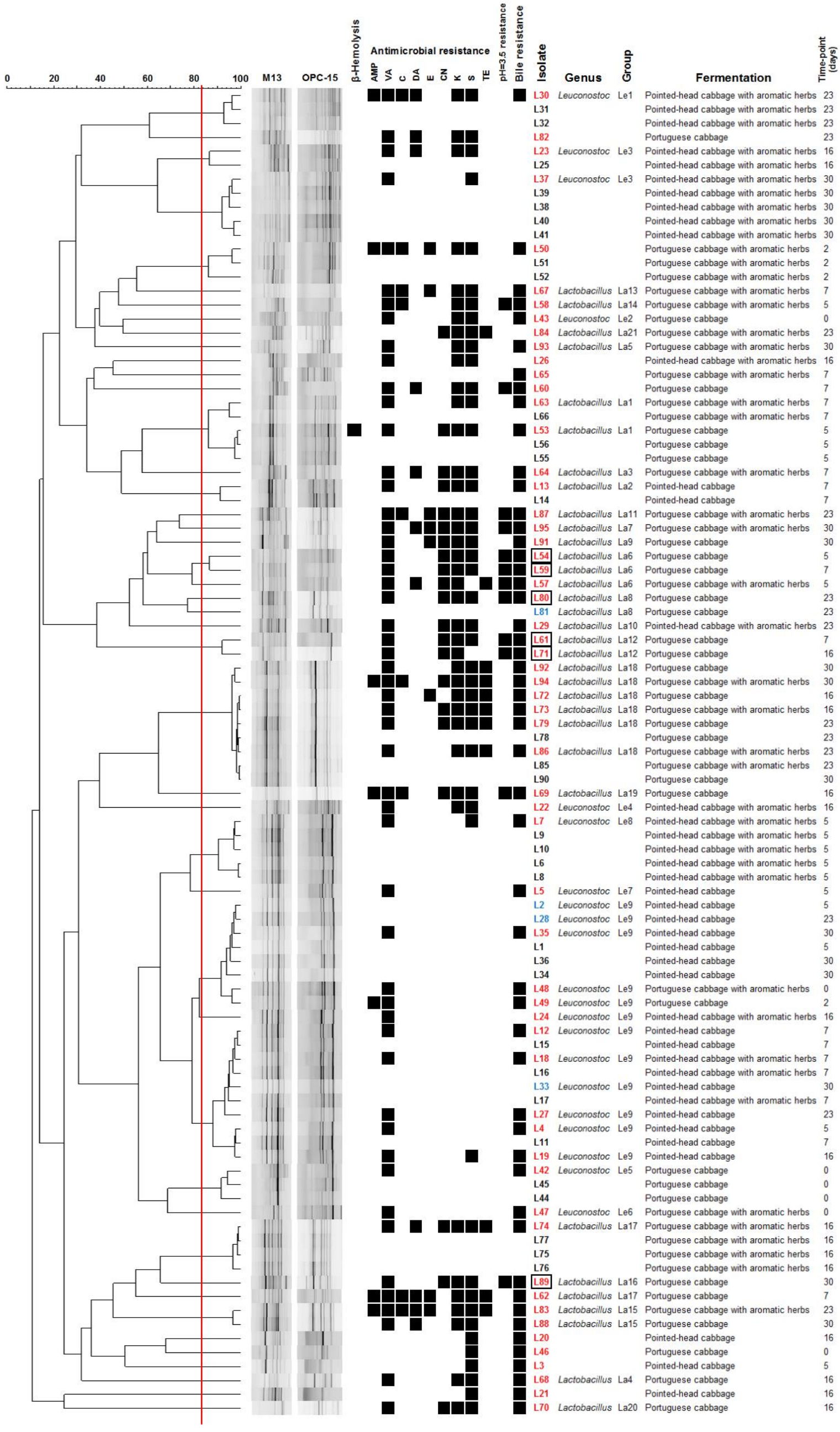

Figure 12 – Summary of results for the 95 isolates with a LAB-like phenotype. Dendrogram constructed based on the PCR-fingerprinting profiles, with the red vertical line representing the reproducibility level. Information regarding hemolytic activity, antimicrobial resistance, low pH (3.5) and bile resistance, genus identification, group attributed after PCR-fingerprinting analysis and isolation substrate is also shown. Isolates written in red were chosen as representatives after PCR-fingerprinting, and isolates written in blue were also chosen, but excluded after further analysis. Isolates marked with a box were selected for subsequent analysis based on the information presented in the figure. Black box - presence of characteristic; AMP - Ampicillin; VA - Vancomycin; C - Chloramphenicol; DA - Clindamycin; E - Erythromycin; CN - Gentamicin; K - Kanamycin; S - Streptomycin; TE - Tetracycline.

all the tested isolates against *Lis. monocytogenes*, which further indicates their probiotic potential. However, the precise mechanism by which this effect occurs is still not understood.

The six isolates were also analyzed for resistance to a lower pH value (pH=2.5) than the one previously evaluated, using a broth based assay. While four isolates were culturable after 3 h of incubation, only three (L54, L61 and L89) were still culturable after 24 h.

Results from other studies regarding acid resistance vary greatly, and this is probably due to differences between methodologies, such as the type of solution used for testing (culture media or non-nutritive buffer), pH values studied (2 to 3) and incubation time (3 to 6 h, but some tested longer times). This high heterogeneity of methods therefore hinders the comparison between results of different studies and the present work.

Although there is no established protocol for assessing resistance to low pH, the agar based methods used in the present study provided a quick methodology to select a small number of isolates with probiotic potential for further testing. The broth based method allowed to further assess this characteristic, and the fact that three of the six selected isolates were resistant to a pH value as low as 2.5 is a good indicator of their suitability as probiotic candidates. Nonetheless, conditions closer to those found in the GI tract, such as the presence of digestive enzymes, should be tested for confirmation.

3.4 - Evaluation of pathogenic and probiotic characteristics of selected isolates using the *Caenorhabditis elegans* model

Two of the three probiotic candidates, L61 and L89, were chosen for testing using the *C. elegans* model. Experiments were performed in two different phases. First, worms were fed with one of four microorganisms: i) a non-probiotic control strain, *E. coli* HT115; ii) a probiotic control strain, *L. rhamnosus* GG (LGG); and iii) the two probiotic candidates (conditioning phase). At this point, the conditioning strain was the only available food resource given to *C. elegans* until they reached adulthood. Once in the adult stage, worms were subjected to a diet of either a pathogenic (*Serratia* sp.) or a non-pathogenic strain (*E. coli* OP50) (testing phase).

Two main parameters were assessed during the testing phase, fertility and longevity of nematodes. Fertility indicates the number of descendants (progeny) per adult worm, whereas longevity corresponds to the number of days that a worm lives and therefore to its survival. In addition to these parameters, quantification of LAB and other bacteria present in the nematode gut was performed throughout the testing phase.

For result analysis, two groups were considered, corresponding to worms fed with *E. coli* OP50 or *Serratia* sp. Comparison of the four different conditionings within the *E. coli* OP50 group allowed the observation of the effects of the candidate probiotic strains in the absence of the pathogen. Comparison of the results of the *Serratia* sp. group with the *E. coli* OP50 group served as a measure of the protective effect of each conditioning strain in an infection context.

Regarding fertility, results showed that there was no significant difference when comparing worms within the *E. coli* OP50 group. The same was found for the worms fed with *Serratia* sp. (**Figure 13**). However, when these two groups were compared to each other, it was evident that feeding with *Serratia* sp. significantly decreased the worm progeny in comparison with feeding with *E.coli* OP50 (P<0.001).

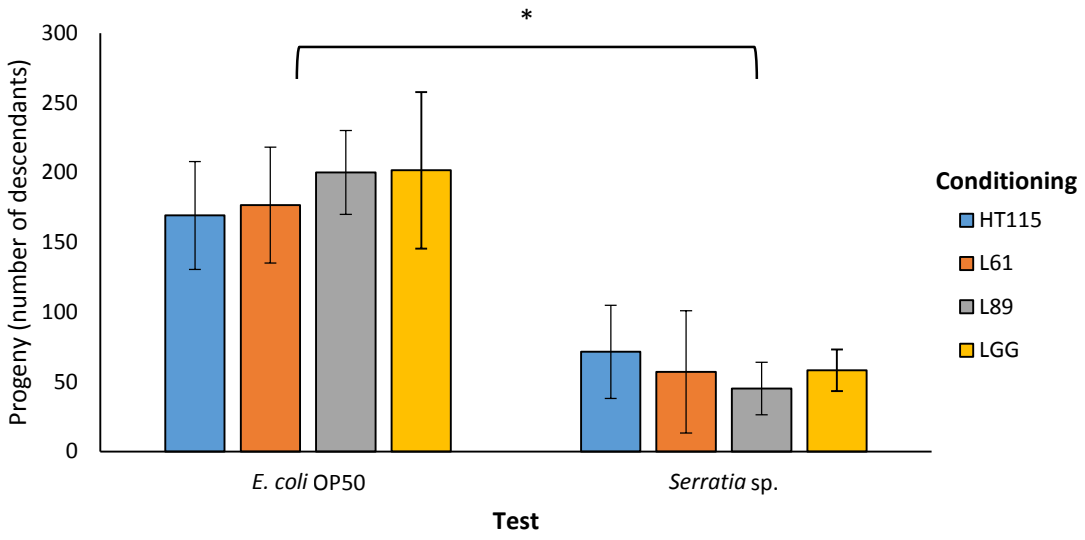

Figure 13 - Effect of different conditioning and feeding test combinations on the total fertility of *C. elegans*. HT115 – *E. coli* HT155; LGG – *L. rhamnosus* GG. * - P<0.001

The distribution of *C. elegans* fertility throughout adulthood was also analyzed. Only conditioning with isolate L61 caused a significant difference in this distribution when nematodes were later fed with *Serratia* sp., compared to those fed with *E. coli* OP50 (P<0.001); with the first group having a higher percentage of their total viable progeny in the first day of testing (**Figure 14**).

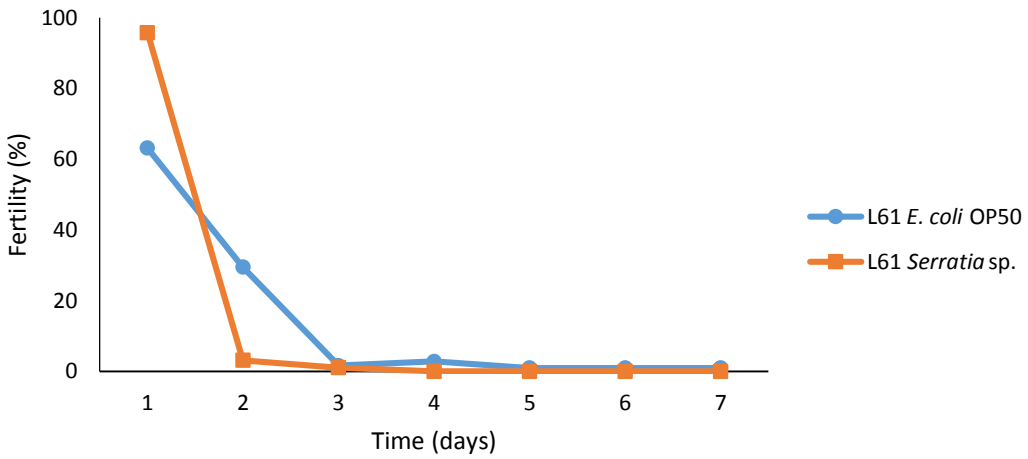

Figure 14 - Distribution of *C. elegans* fertility when conditioned with L61 and then fed with either *E. coli* OP50 or *Serratia* sp.

Considering nematode longevity, and survival in the absence of pathogens (**Table 6**), results showed that, for the *E. coli* OP50 group, none of the different probiotic conditionings increased longevity or significantly reduced risk of death compared to the non-probiotic control conditioning, with only nematodes in contact with isolate L89 and LGG showing a slight, non-significant reduced risk of death. On the other hand, conditioning with isolates L61 and L89 led to a significantly increased risk of death on worms fed with *Serratia* sp., in comparison with those fed with *E. coli* OP50 (4.5-fold, P<0.005, and 3.3-fold, P<0.05, respectively). Significantly increased risk of death was not observed for conditioning with *E. coli* HT115 and LGG.

Table 6 - Survival results for nematodes submitted to different combinations of conditioning and testing. Mean lifetime indicates the observed time at which 50% of the nematodes were dead. Hazard ratio indicates the proportion of estimated risk of death of an individual in comparison to the appropriate control group: groups fed with *E. coli* OP50 were compared to the *E. coli* HT115 + *E. coli* OP50 treatment, while groups fed with *Serratia* sp. were compared with the group conditioned to the same microorganism but fed with *E. coli* OP50.

Treatment	Mean lifetime (days)	Hazard ratio
HT115 + OP50	9.0	-
HT115 + *Serratia* sp.	7.0	1.33
L61 + OP50	8.0	1.24
L61 + *Serratia* sp.	5.5	4.48(**)
L89 + OP50	7.0	0.98
L89 + *Serratia* sp.	7.0	3.30(*)
LGG + OP50	9.0	0.83
LGG + *Serratia* sp.	7.0	2.47

HT115 – *E. coli* HT155; LGG – *L. rhamnosus* GG; OP50 – *E. coli* OP50; * - P<0.05; ** - P<0.005

Finally, regarding the presence of viable microorganisms in *C. elegans* gut, at the beginning of the testing phase it was not possible to isolate LAB strains from worms conditioned with LGG and low quantities were isolated from worms conditioned with L61 and L89. It was not possible to further isolate LAB strains along the experience. On the other hand, microorganisms fed during this phase (*E. coli* OP50 and *Serratia* sp.) were successfully isolated from the nematode gut.

Globally the results allowed several conclusions. First, the *Serratia* sp. strain used in the present work had a clear pathogenic effect on *C. elegans*, reducing its fertility and leading to a reduced mean lifetime. These results are in agreement with previous findings, which showed that *Serratia marcescens* leads to infection in *C. elegans*, with worms showing signs like altered rate of egg-laying and early death, starting three days after contact (Mallo *et al.*, 2002), and confirm the applicability of the strain as a pathogenic indicator. In addition, *Ser. marcescens* is an opportunistic pathogen in humans, associated with nosocomial infections and considered an ongoing public health challenge due to the intrinsic antimicrobial compound resistance of many strains (Marsh and May 2012), and therefore can serve as a human reference pathogen.

On the other hand, conditioning with the probiotic strain LGG did not produce a longevity-increasing or protective effect on nematodes when tested with either *Serratia* sp. or *E. coli* OP50, and in fact even induced an increased, albeit not significant, risk of death when these were fed with *Serratia* sp. LGG has been used as a probiotic control strain in a study involving the *C. elegans* model which found that, contrary to this work, contact with LGG significantly increased the longevity of worms compared to the negative control, although its protective effect against pathogenic infection was not studied (Zanni *et al.*, 2015). Because of these conflicting results, it was not possible to establish the probiotic effect of the candidate strains L61 and L89.

Conditioning with probiotic candidates proved to be safe when nematodes were not in subsequent contact with the pathogen, showing no negative effect both in terms of fertility and risk of death. On the contrary, when these worms were then fed with *Serratia* sp., negative effects were observed. First, testing with *Serratia* sp. after conditioning with isolates L89 and L61 led to an increased risk of nematode death; the latter also induced a change in the behavior of the worms in terms of fertility. This indicates that the isolates had a synergistic effect with *Serratia* sp., enhancing its pathogenic effects on *C. elegans*. However, since the LAB isolates were not found in the gut of the worms, it is likely that this effect is not due to their direct interaction with the pathogen. One possible explanation is that L89 and L61 have a non-detectable debilitating effect on *C. elegans*, which leads to a higher vulnerability when the pathogen is then present.

It is important to note that the potential of the tested LAB strains to be used as probiotics should not be discarded, since they might still have a protective effect against other microorganisms, such as Gram-positive bacteria. This is supported by a previous study which showed that conditioning with a probiotic *Lactobacillus acidophilus* strain led to increased protection against Gram-positive but not Gram-negative microorganisms (Kim & Mylonakis, 2012). Therefore, other pathogenic microorganisms should be included in further assays regarding the probiotic potential of these candidates. Furthermore, other *in vivo* models, such as mice, may be used to assess and establish probiotic effects and should also be considered in future studies.

LAB tested using the *C. elegans* model have commonly been reported as extending the lifespan and/or protecting the worms from pathogenic infection (Grompone *et al.*, 2012; Ikeda *et al.*, 2007; Kim & Mylonakis, 2012; Lee *et al.*, 2011; Park *et al.*, 2014; Zhou *et al.*, 2014), with the exception of *Ent. faecalis*, which can establish infection and lead to nematode death (Garsin *et al.*, 2001; Marsh & May, 2012). On the other hand, Fasseas and coworkers (2013) observed that exposure to three different LAB strains had a lifespan reducing effect in adult nematodes, although they did not test their protective effects against pathogenic infection. It should be noted that the methods used in the present work were somewhat different than those from other works. While other studies expose the nematodes to LAB strains in early adulthood, here nematodes were exposed from birth until the beginning of adulthood, in order to study the fertility of adult worms. Furthermore, time of exposure to these strains also varied in the other studies. These differences could explain conflicting results, including the non-protective effect of LGG.

The synergistic effect of LAB with pathogens has not been previously reported, and while it is not a definite excluding trait, it raises the possibility that these isolates should not be applied as probiotic microorganisms. Nonetheless, more studies should be performed in order to correctly establish the potential of these microorganisms as probiotics.

4 - Conclusions

The main objective of this work was the isolation of LAB from sauerkraut fermentations and the selection of candidates with the highest probiotic potential. For this purpose 18 sauerkraut fermentations were performed and four were chosen for further studies. These showed a similar evolution of pH, acidity and LAB quantity over time when compared to other studies described in the literature, which suggests that the fermentations were performed correctly. From these four fermentations, 114 microorganisms were isolated, 95 possessing a LAB-like phenotype.

Afterwards, the 95 isolates were characterized genotypically by PCR-fingerprinting, which allowed the selection of representative isolates, followed by identification to the genus level, with the majority being identified as *Lactobacillus* spp. or *Leuconostoc* spp. The analysis of these results also allowed the study of the diversity and the distribution of LAB throughout the four fermentations, with the conclusion that LAB diversity and composition depended on the type of cabbage used as substrate for sauerkraut production.

Representative isolates were then studied as to their safety and probiotic features. One isolate presented β-hemolytic activity, and 42% showed antimicrobial resistance associated with transmissibility, characteristics that may be unsafe and not desirable in probiotic microorganisms. Regarding their probiotic potential, most isolates were resistant to the presence of bile, but few were able to resist low pH conditions. The six isolates that were able to resist both conditions and were previously considered safe were further tested, and found to possess antimicrobial activity against *Listeria monocytogenes*. Furthermore, three were able to resist lower pH conditions, and were selected as having the best probiotic potential.

Two candidates were then tested using the *C. elegans* model, with results showing no protective effect on the nematodes. On the other hand, contact with these isolates led to increased pathogenesis of infection with *Serratia* sp., despite no evidence of detrimental effects on nematode health when the pathogen was not present. This indicated that these microorganisms may not be appropriate for use as probiotics, and should be further characterized.

To conclude, this work reached its intended goal, resulting in the isolation of three potential probiotic strains from sauerkraut fermentations. Although two of these did not show probiotic characteristics in an animal model, further work should be performed to characterize them, including the evaluation of other important characteristics such as GI tract adhesion, testing their protective effect against other pathogens, evaluation of the remaining strain in the *C. elegans* model, and further testing in more complex animal models, in order to clarify their potential as probiotic microorganisms.

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Appendix A – List of all isolates obtained for the four tested fermentations

Table 1 – List of all isolates obtained for the four tested fermentations. Isolates attributed with a simplified code were selected for further studies after phenotypic characterization.

Isolate	Simplified code	Fermentation substrate	Time-point (days of fermentation)
3CS5L180	L1	Pointed-head cabbage	5
3CS5L181	L2		
3CS5L182	L3		
3CS5L183	L4		
3CS5L184	L5		
3CE5l185	L6	Pointed-head cabbage with aromatic herbs	5
3CE5l186	L7		
3CE5l187	L8		
3CE5l188	L9		
3CE5l189	L10		
3CS7L195	L11	Pointed-head cabbage	7
3CS7L196	L12		
3CS7L197	L13		
3CS7L198	L14		
3CS7L199	L15		
3CE7L200	L16	Pointed-head cabbage with aromatic herbs	7
3CE7L201	L17		
3CE7L202	L18		
3CE7L203	-		
3CE7L204	-		
3CS16L269	-	Pointed-head cabbage	16
3CS16L270	-		
3CS16L271	L19		
3CS16L272	L20		
3CS16L273	L21		
3CE16L274	L22	Pointed-head cabbage with aromatic herbs	16
3CE16L275	L23		
3CE16L276	L24		
3CE16L277	L25		
3CE16L278	L26		
3CS23L339	-	Pointed-head cabbage	23
3CS23L340	-		
3CS23L341	-		
3CS23L342	L27		
3CS23L343	L28		
3CE23L344	L29	Pointed-head cabbage with aromatic herbs	23
3CE23L345	-		
3CE23L346	L30		
3CE23L347	L31		
3CE23L348	L32		
3CS30L384	L33	Pointed-head cabbage	30
3CS30L385	-		
3CS30L386	L34		
3CS30L387	L35		
3CS30L388	L36		
3CE30L389	L37	Pointed-head cabbage with aromatic herbs	30
3CE30L390	L38		
3CE30L391	L39		
3CE30L392	L40		
3CE30L393	L41		
4PS0L236	L42	Portuguese cabbage	0
4PS0L237	L43		
4PS0L238	L44		
4PS0L239	L45		
4PS0L240	L46		
4PE0L241	L47	Portuguese cabbage with aromatic herbs	0
4PE0L242	L48		
4PE0L243	-		

Isolate	Simplified code	Fermentation substrate	Time-point (days of fermentation)
4PS2L289	-	Portuguese cabbage	2
4PS2L290	L49		
4PS2L291	-		
4PS2L292	-		
4PS2L293	-		
4PE2L294	L50	Portuguese cabbage with aromatic herbs	2
4PE2L295	L51		
4PE2L296	L52		
4PE2L298	-		
4PS5L309	-	Portuguese cabbage	5
4PS5L310	L53		
4PS5L311	L54		
4PS5L312	L55		
4PS5L313	L56		
4PE5L314	L57	Portuguese cabbage with aromatic herbs	5
4PE5L316	L58		
4PS7L329	L59	Portuguese cabbage	7
4PS7L330	L60		
4PS7L331	L61		
4PS7L332	L62		
4PS7L333	-		
4PE7L334	L63	Portuguese cabbage with aromatic herbs	7
4PE7L335	L64		
4PE7L336	L65		
4PE7L337	L66		
4PE7L338	L67		
4PS16L409	L68	Portuguese cabbage	16
4PS16L410	L69		
4PS16L411	L70		
4PS16L412	L71		
4PS16L413	L72		

Isolate	Simplified code	Fermentation substrate	Time-point (days of fermentation)
4PE16L414	L73	Portuguese cabbage with aromatic herbs	16
4PE16L415	L74		
4PE16L416	L75		
4PE16L417	L76		
4PE16L418	L77		
4PS23L424	L78	Portuguese cabbage	23
4PS23L425	L79		
4PS23L426	L80		
4PS23L427	L81		
4PS23L428	L82		
4PE23L429	L83	Portuguese cabbage with aromatic herbs	23
4PE23L430	L84		
4PE23L431	L85		
4PE23L432	L86		
4PE23L433	L87		
4PS30L449	L88	Portuguese cabbage	30
4PS30L450	L89		
4PS30L451	L90		
4PS30L452	L91		
4PS30L453	L92		
4PE30L454	L93	Portuguese cabbage with aromatic herbs	30
4PE30L455	-		
4PE30L456	L94		
4PE30L457	L95		
4PE30L458	-		